

Israel's nuclear weapons in the open

The US plan to contain the accumulation of destructive weapons in the Middle East has much to commend it, but it will require that Israel should be open about its nuclear weapons.

ISRAEL, a crypto-nuclear power since the early 1960s, will probably be flushed out into the open on that issue by the arms control initiative for the Middle East announced last week by President George Bush. Israel's nuclear complex at Dimona, in the Negev desert, has no known functions except the training of nuclear engineers and the manufacture of modest amounts of plutonium, which over the years would have allowed the manufacture of some scores of atomic bombs. The Bush proposal is that there should be an agreement among the major arms suppliers not to provide the ingredients of 'weapons of mass destruction' to states in the Middle East and North Africa, accompanied by an externally monitored 'freeze' on the manufacture of nuclear weapons. It is a good idea. There is a plan that the five governments which are permanent members of the United Nations Security Council should meet in Paris early in July to see if agreement can be reached.

On one reading of the strategic situation, Israel has the strongest possible reason to be a nuclear power. It is at least technically at war with most of its neighbours (the exception is Egypt) and evidently vulnerable to conventional attack — well-armed tanks could drive from Jerusalem to the Mediterranean coast in half an hour. So what better means of avoiding military catastrophe than to threaten the retaliatory destruction of the perpetrator's capital and other population centres? This is classical deterrence. Moreover, it hardly matters whether the threat is open or implicit. Even as things are, any neighbour planning a serious attack on Israel would have to calculate the risks of nuclear retaliation.

Israeli governments, which are never fools, have studiously fostered uncertainty on the issue. Israel has not signed the Nuclear Non-Proliferation Treaty (NPT). Government statements on the nuclear question blend a refusal to forswear military options that may make future sense with a refusal to say what is the present state of affairs. The result is that most people, including neighbour governments, believe that Israel has a modest stock of nuclear weapons — but neighbour governments are denied the sense of indignation that sure knowledge would give them, not to mention the right to make claims on states elsewhere that might be induced to lend them nuclear assistance. That, no doubt, explains why Iraq, Libya and Pakistan have all, from time to time and with more or less reason, headed the list of potential nuclear proliferators.

Bush's proposal would remove some of that uncertainty. And, ironically, Israeli commentators have already reacted

by arguing that a freeze on nuclear weapons in the Middle East would be unfair to Israel when nuclear forces are the only valid counterpoise to the potential preponderance of conventional forces that it faces. But what if the dangers posed by those forces should be substantially reduced, perhaps by an agreement among the chief arms suppliers? Then, Israel's need of a nuclear deterrent, even a hypothetical deterrent, would in logic be reduced. Indeed, on a less narrow view of Israel's present danger than that habitually taken by Israeli governments, the need for the capacity of nuclear retaliation has also been eroded by the recent demonstration of the mobility of US conventional forces, likely only to be reinforced by the decision of the United States to base substantial stockpiles of military equipment in Israel itself.

That is why Israel has nothing to lose by, first, acknowledging that it is a nuclear power and, second, by allowing that it would itself be safer if it were to trade its status as a nuclear power for arrangements making sure that none of its natural enemies could follow suit. It should now be all the safer. What seems to have changed, partly no doubt as a consequence of Mr James Baker's dispiriting merry-go-round between the capitals of the Middle East, is that the United States is reconciled to becoming the de facto physical guarantor of Israel's continued existence if a regional peace settlement cannot be reached. There are many in Israel who will not trust that development, let alone welcome it. But they have no choice.

For people elsewhere, the past ten months (since the invasion of Kuwait) have mostly been dispiriting. At one stage, it seemed as if the superpower agreement on conventional weapons was about to fall apart, while the ending of an apparently successful war to undo the invasion has been followed by hardly any tangible sign that similar events will not recur.

But recalcitrant Middle East states have left out of their calculations that states elsewhere are as fearful that the region will remain a tinderbox as they should be themselves — and that they have the power to make things otherwise. That (as well as the hope of joining this year's Western economic summit) seems to have prompted the change of heart in Moscow on the conventional arms treaty. France (the ultimate originator of the concept of arms control "from the Atlantic to the Urals") seems about to set Israel an awkward example, as it talking of joining the NPT. Maybe not all is lost. □



Gallo vs Montagnier?

Doubts remain about the origins of the AIDS virus, despite the certainties of the press in the past few days.

THE most bitter rows seem never to come to an end of their own accord. That seems to be the lesson to be learned from the fuss generated in the general press by the publication last week of a brief letter from Dr Robert C. Gallo of the US National Institutes of Health (NIH). Both in Europe and the United States, the newspapers have once again rushed to an adjudication of who is right, and who is — by extension — wrong in his claims for precedence for the AIDS virus, now called HIV (human immunodeficiency virus): Gallo or Dr Luc Montagnier, at the Pasteur Institute? These adjudications have mostly been unfair to one or other of the participants, perhaps even to both. It is in the public interest that there should be a better understanding of what the controversy is about.

First, there is the question of whether the virus used by Gallo and his colleagues in 1984 had been derived not from a locally collected sample, but from one sent from the Institut Pasteur in Paris. This possibility was most tangibly suggested by the publication of the complete nucleotide sequences of the two viruses early in 1985. Second, there have been suggestions that the close similarity of the two viruses is not an accident, but the result of the deliberate misuse of the French virus by somebody in Gallo's laboratory. Virology being what it is, cross-contamination of viral strains is commonplace. The second allegation against Gallo and his colleagues is much more serious. The NIH have stated that, in an inquiry now more than a year old, they have found no evidence of theft and no motive. But the NIH continue their investigation into the details of some of Gallo's papers. No report has been forthcoming yet.

The developments in the past few weeks which have thrown light on the origin of HIV are chiefly two. First, Gallo and his colleagues had the wit to go back to their freezers and to use techniques not available in 1983 (when the hunt for a virus began in earnest) to work out the genetic structure of the viruses with which they were then working. Their first report (*Nature* 349, 745; 1991) seemed clear enough — among the early samples sent to Bethesda from Paris, two out of three differed from both the French and the US sequences published in 1985. The third sample, the receipt of which was acknowledged, could not be found. So it seemed that Gallo's virus could not have been derived from the French and that even the French sequence could not have come from the 1983 samples supposed to contain it. Gallo's corrections of his estimates of the significance of the observed differences (*Nature* 351, 358; 1991) does not affect this inference substantially.

The second development was the publication, a few weeks ago, of Montagnier's parallel investigation (*Science* 252, 961; 1991). From that, two things emerge. First, the third of the French samples, no longer to be found in Gallo's freezer, contains not one, but two, viruses. Montagnier and his col-

leagues give an explanation of how the first virus may have been contaminated by the second. They give chapter and verse for their belief that their cultures had already been contaminated in Paris by the time a sample was sent to Gallo. And, second, Montagnier and his colleagues show that contaminating virus (from a patient with the initials LAI) is for practical purposes identical with that eventually published by them and with the published sequence of Gallo's virus and with some of the strains used in his laboratory in late 1983 and early 1984. The significance of these developments was explained two weeks ago (*Nature* 351, 267; 1991).

The implications require very little elaboration. First, on the question whether Gallo's virus was contaminated by Montagnier's, barring whatever surprises may emerge from further analyses of the contents of other people's freezers, the answer seems to be 'Yes'. For many of the past 12 months, Gallo has been admitting the possibility, and has now done so openly.

Second, on the question whether Gallo and Montagnier can be said to have discovered the virus now called HIV independently of each other, much hangs on the meaning of 'discovery'. If that means the recognition of a novel virus by means that do not prove its viral character (by electron microscopy, for example), the credit has been known for years to go to Montagnier. But those in the trade would rather hold to something like Koch's first postulate, that an entity claimed to be a virus must be capable of infecting other cells in such a way that it can be recovered from them (so as to reinfect others cells, and so on...). By that test, it hardly matters what sample is used at the outset, provided that the demonstration of productive reinfection is cast-iron. In that case, the credit is shared equally, although it was Gallo's laboratory that first got large-scale production for a blood test.

The third question, that of whether somebody in Gallo's laboratory knowingly misused the French virus samples to make a usable tool in the then new battle against AIDS is literally unaffected by the essence of what has recently been published. In the circumstances, it is wrong that Gallo should be judged guilty of all offences in the calendar simply because one strain of virus may have contaminated his own cultures, as it had already done Montagnier's. Whatever happened to the presumption of innocence? Gallo's plea last week that the time has returned for collaboration between two important research groups deserves a fair hearing. There is no evidence that Gallo stole Montagnier's virus, but he may have stolen the limelight. □

Dispensable carnival

The annual international AIDS conference has outlived its usefulness and should be stopped.

SHOULD the annual international conference on AIDS continue? Later this month, thousands of researchers and health workers will descend on Florence for this year's jamboree. (The venue alternates between the United States and somewhere else in successive years.) Last minute participants will

be scrambling for hotel rooms in a city that has been fully booked for more than a year. But AIDS activists in the United States are doing everything they can to ease the accommodation problem by demanding a decrease in the size of the delegation from the US Department of Health and Human Services, which includes the National Institutes of Health and the Centers for Disease Control, among other agencies. In the short tradition of these conferences, a pre-conference row is par for the course, but this year's is sillier than most.

Armed with the information that the department plans to spend \$1.5 million of government funds to send 392 people to Florence, AIDS activists have enlisted the help of members of Congress in mounting a protest. They argue that the money could be better spent treating patients who are dying of the immunodeficiency disorder — subsidizing AZT for patients unable to pay, for instance. The department has answered that "everyone who is going has a worthwhile" reason. Really? Three hundred and ninety-two worthwhile reasons? Nobody would wish to deprive US researchers and health workers of a sight of the city that cradled the Renaissance, but the department's number is large enough to suggest that there could be a worthwhile conference if those concerned simply stayed at home. That is the cogent point the AIDS activists have grasped.

The protest goes off the rails by equating economies in the travel budget with the improved treatment of AIDS. The dollars saved even by cutting the official US delegation in half would make only a minor dent in the need for better care of AIDS patients. One is forced to the conclusion that the activists are not seeking to make a serious argument, but a debating-point. In doing so, they have overlooked the harm that would be done by a precedent that would have congressmen and activists dictating who attends a scientific meeting.

Those seeking to throw a spanner in the works should take a more direct line, starting from the plain truth that annual AIDS conferences have become unwieldy, unmanageable and unproductive. The original idea was that the world would hugely benefit from regular public reviews of the progress it was hoped researchers would make in tackling the public health problem by which the 1980s will be remembered. But hope has not been matched by reality. Confident diagnosis is possible, there is a single drug that can be used to slow the progress of the disease and the management of patients has been much improved. But there is no cure or even sure prophylactic in sight.

That is the chief reason why only bad temper multiplies at these conferences. Researchers have a huge volume of new work to review each year, but there is not much to lift the hearts of the camp followers who scamper from one parallel session to another. So frustration abounds, driving the activists to protest that their interests are being neglected when the truth is that their constituents are the poignant victims of a problem of public health most obviously characterized by its obduracy. These are not the circumstances in which there can be a constructive assessment of what research has done. Serious researchers have been saying so for years, and many already save their most significant data for smaller conferences,

where the chances for real discussion are better.

The organizers should listen carefully, but urgently, to what they are saying. It is time to re-evaluate this carnival. And meanwhile, it is safe to predict that next year's pre-conference row will be a re-run of last year's. Last week, the US Department of Justice decided to override the opinion of the Department of Health and Human Services, which had taken the sensible view that infection with HIV should not be a reason for banning a person's entry to the United States. The Department of Justice is responding to public anxiety, not to the realities of what is known of the infection. Such obscurantism is also a bad precedent. □

Candidate for change

The first Democratic candidate for US president would make technology his standard.

RECENT history has taught us not to expect much serious debate in the course of US presidential campaigns, which seem to have fallen victim to the 30-second sound bites of the television age. So it is a welcome relief that the first man to throw his hat in the ring as a presidential candidate for the Democratic party in November 1992 plans to run on a platform grounded in science and technology.

Former US Senator Paul E. Tsongas of Massachusetts has written an 85-page campaign statement declaring that the future economic strength of the United States lies in the 'creation of national wealth' through a new manufacturing base and a strong commitment to research. In *A Call to Economic Arms*, he says that the manufacturing base of the United States should be for the 1992 campaign, noting that Germany has 33 per cent of its workforce in manufacturing, Japan 28 per cent and the United States only 17 per cent, down from 26 per cent in 1970. For a Democratic candidate, he also takes a radical stand when he declares that "corporate America must survive", if only on the grounds that a party committed to the redistribution of wealth 'cannot redistribute wealth that is never created'.

Tsongas's solutions are two. First, he would bite the bullet on the issue of a national industrial policy, with the government making strategic investment decisions in areas including ceramic engines, supercomputers and memory chips. This is campaign rhetoric never heard before. Second, Tsongas would focus resources on the National Institutes of Health, the National Science Foundation, the National Aeronautics and Space Administration and the Departments of Energy and Agriculture, saying the United States should not be satisfied with marginal budget increases. He calls it a matter of 'mindset.' "The Manhattan Project. The Apollo program. The war in the Persian Gulf. It's just a matter of recognizing the threat and responding to it."

The significance of Paul Tsongas's platform is its seriousness. Here is a man trying his best to cast the presidential debate in terms of issues that really matter. He is on the right track. If he can elevate the debate, he will have performed an important service. He certainly deserves thanks for trying. □

Japan, Europe lobby US over space station

■ Possible cancellation causing alarm

■ Future collaborations could be affected

Tokyo, London & Munich

As the US House of Representatives was debating the fate of the planned US Space Station this week, scientists and government officials from Japan and Europe were doing their best to see that the ambitious programme stays on track.

Because Japan and Europe have already spent hundreds of millions of dollars developing their own contributions to the station, a cancellation at this point would be a stinging lesson that the United States cannot be trusted to keep its scientific commitments, said foreign officials from several countries. A decision to abandon the space station would almost certainly kill any hope there might be of Japanese contribution to the US Superconducting Super Collider (SSC), and certain European countries could reduce or halt their cooperation in collaborative space ventures with the United States.

On Monday this week, the future of the Space Station was still up in the air. On 15 May, a House subcommittee voted to discontinue funding the station, and a vote in the full House was expected to take place today (6 June). But even if the House votes to cancel the station, the Senate is likely to put funds for it back into the budget, and the matter would then go to a conference committee for resolution. A number of observers have said that the Space Station's fate probably rests with President Bush — it will be saved only if he pushes hard for it.

The Japanese have been the most vocal protesters, both because they have the most to lose and because they seem likely to have the most leverage on US science policy. Japan's Science and Technology Agency (STA) has already invested ¥40,000 million (about \$300 million) in developing the Japanese Experimental Module for the station — the module is expected to cost about \$2,000 million when complete — and the station's share of STA's space budget has been growing rapidly year by year. This year it will absorb ¥18,000 million, or 14 per cent of the science agency's ¥130,000 million budget. A cancellation of the space station would be disastrous for Japan's space programme.

Furthermore, Japan appears to be the only country that might make a major contribution to the SSC. US officials hope to persuade Japan to pick up \$1,000 million of the projected \$8,250 million cost of the project.

In the past two weeks, two senior Japanese officials have sent three strongly worded letters to their counterparts in the United States suggesting that abandonment of the space station would severely damage the credibility of the United States in future

international "big science" projects. The Japanese say they were encouraged to send the letters by US Administration officials, who are trying to push the station's budget through Congress.

Shigeo Iwatani, director of the division of scientific affairs at Japan's foreign ministry, says that soon after the subcommittee's vote to cancel funds for the space station, Japanese Embassy officials in Washington met representatives of National Aeronautic and Space Administration (NASA) and the State Department. "Both sides thought it better for Japan to express our concerns in writing" to help the US administration win back funding for the project, Iwatani says.

On 24 May, Taro Nakayama, head of Japan's Ministry of Foreign Affairs, wrote a letter to US Secretary of State James Baker. This was quickly followed three days later by two letters from Akiko Santo, head of Japan's Science and Technology Agency, to NASA administrator Richard Truly and to White House science adviser D. Allan Bromley. All the letters are unusually blunt by Japanese standards and similar in content.

Santo's letter makes three main points, according to Shinichiro Ogura, director of the science agency's office of space utilization. First, since accepting the US invitation in 1984 to join the project, Japan has been making "diligent efforts" to modify its national space policy and has invested "quite large resources" in preparing for the space station, but all of this will be nullified if the project is abandoned. Second, Japan and the other international partners have worked hard to restructure the space station project to meet new requirements imposed by Congress, and Japan finds it "very difficult to understand what is now taking place in Congress". Third, the space station is symbolic of US-Japan collaboration in science and if the project does not proceed smoothly, "doubt would inevitably grow about the credibility of the United States as a partner in international collaboration."

This doubt would inevitably make Japan harder to entice into other collaborative scientific projects. At this point, for instance, Japan's various science-related agencies and ministries have reached no agreement on how to deal with the SSC request. The required funds are far beyond the budgetary capabilities of any one ministry or agency. But the two most appropriate organizations to support the project, the STA and the Ministry of Education, Science and Culture, are constantly at loggerheads over how to divide up Japan's basic research budget.

The education ministry is trying to improve Japan's rundown universities and would prefer to spend the science funds on such domestic issues. A US decision to abandon the space station would almost certainly tip the scales against a Japanese contribution to the SSC.

The threatened cancellation of the space station is also causing alarm in Europe. The European Space Agency (ESA) has already spent nearly \$1,000 million — spread among several countries — on the Columbus programme, Europe's contribution to the space station. Columbus consists of a manned microgravity laboratory which will be bolted on to the station, a free-flying unmanned laboratory, and the polar platform, which is a large remote-sensing satellite. Cancellation of the space station would kill the manned laboratory, although the other two components could continue.

Franco Emiliani, manager of the Columbus programme, says the uncertainty is undermining the morale of project scientists. "It's a very annoying situation."

ESA itself, however, can exert little political pressure over the US decision. Because the space agency represents 13 separate member states, any effort to threaten the United States with reprisals would require an unprecedented degree of international cooperation.

Germany would wield the most influence of any European country. As the project leader on the Columbus, with a 38 per cent share of the costs, it has the most to lose from a cancellation of the space station. Between industry and government money, Germany has already invested about DM500 million (\$294 million) in Columbus.

If the United States cancels the space station, says Klaus Berge of the German government space agency DARA in Bonn, it would be seen as a breach of an international contract that would certainly have consequences for German-US cooperation. The first thing politicians in Germany would do, Berge says, would be to check all other cooperative programmes to see where they might retaliate. The first ones that come to mind, he says, are the ROSAT X-ray satellite, which is currently gathering data for a US project, and the CRAF mission to investigate a comet up close. But cooperation on the SSC — where Germany could help to build detectors for the collider — would also be endangered, Berge says.

But Germany would not be as free as Japan to retaliate against the United States, Berge says, because Germany has its own space project — the D2 spacelab mission scheduled to fly on the space shuttle in 1992 — which is dependent on US cooperation.

The House Committee on Science, Space and Technology, which supports the space station, was scheduled to hold hearings on the international consequences of cancellation on 4 June.

David Swinbanks, Peter Aldhous & Steven Dickman

BOOK PRESERVATION

Library to deacidify books

Washington

THE Eisenhower Library at Johns Hopkins University in Baltimore is starting an innovative programme to save thousands of books threatened by the acid in their pages. At a cost of nearly \$11 per volume, the books will be deacidified with a 60 hour treatment in a vacuum chamber filled with diethyl zinc (DEZ) gas.

Officials at Hopkins and at Akzo Chemicals, the company providing the service, say that, as far as they know, this is the first time a library in the United States has undertaken such a mass deacidification campaign.

Most books printed since 1800 have been made with paper that has a high acid content which, over the years, causes the pages to become brittle and eventually crumble into dust. Saving those books is expensive — it can cost \$100 a book or more to copy them onto microfilm, and a custom preservation job in which a book is taken apart and restored page by page costs about \$1,000 a volume.

So, in the mid-1970s, scientists at the Library of Congress began to look for an alternative. They developed a process that uses gaseous DEZ to penetrate the pages of books and react with any acids to form neutral salts. In addition, the DEZ reacts with any residual water in the paper to form zinc oxide, which remains in the paper as a 'buffer' to counteract any acid that attacks the pages in the future. The process was licensed to Akzo, which had supplied the DEZ for the development.

Akzo spokesman Dick Miller says tests indicate that books treated with DEZ will

last three to five times longer than untreated books, which usually have a lifetime of 50 to 100 years.

When the company begins truly largescale programmes, where thousands of books are treated at a time, the cost per book should drop to between \$6 and \$10, he says.

Over the next year, the Johns Hopkins library plans to send about 300 books per month to Akzo, most of them foreign language paperbacks published outside the



West. Scott Bennett, director of the Eisenhower Library, explains that these books tend to be printed in limited printing runs on very acidic paper, so that they are among the hardest books to replace.

Akzo is hoping to land a contract with the Library of Congress, which is under congressional mandate to deacidify millions of books in the coming decade. Although Library of Congress scientists developed the DEZ method, the library is also considering two deacidification processes that use liquid treatment to neutralize the acid in the pages. "Many people (at other libraries) are watching to see what the Library of Congress decides," Miller says.

Robert Pool

SPACE EXPLORATION

Moon base best, says panel

Washington

FROM the rapidly shrinking list of reasons to build the US-led international space station, subtract one more. Given the increasingly likely prospect of a delayed and under-equipped station, a lunar orbiter and a moon base might provide better preparation for the Mars mission now being planned, a blue ribbon panel commissioned by the National Aeronautics and Space Administration (NASA) has concluded.

Scientists could use existing vehicles such as the space shuttle and the Soviet Mir station for life-science research over the next decade or so, according to portions of the panel's report leaked last week. Eventually, researchers could move to stations in lunar orbit and a lunar base in order to study human adaptability to the long-term weightlessness of space travel and the one-third Earth gravity of Mars, the report says.

The panel, headed by former US general Thomas Stafford, was assembled last year to take a fresh look at the NASA's planned

Moon/Mars initiative and to solicit new ideas from outside the government and the usual contractors (see *Nature* 345, 651; 21 June 1990). It intends to release its full report next week at a press conference.

If the space station can be built in time and at full capacity, it would be the preferable platform for much of the life science research, the panel said. But as NASA reduces the station's capacity in an effort to cut costs and fight a threatened congressional cancellation, the panel suggested that "it is logical to consider the Moon" as a viable alternative.

Such faint praise for the space station will hardly improve the job of the NASA officials now fighting to save the troubled project in the face of congressional opposition. Life science research is one of the station's remaining scientific missions. The suggestion that even that could be done well elsewhere leaves NASA with less justification for the \$30,000 million project, congressional staff say.

Christopher Anderson

THREE MILE ISLAND

A stress-cancer link following accident?

Washington

A SMALL wave of excess cancers occurred three years after the 1979 Three Mile Island nuclear accident among people living close to the plant, according to a report published in the *American Journal of Public Health* (81, 719-724; 1991), but the increase in cancers was not caused by radioactivity released during the mishap. Instead, the authors suggest, the culprit could have been either stress or simply increased attention towards health concerns.

The research team, led by Maureen Hatch of the Columbia University School of Public Health in New York City, examined cancer rates after the accident as a function of distance from the plant: less than 6 km, between 6 and 12 km and between 12 and 22 km. Among people in the latter two groups, cancer rates remained unchanged, but the researchers found an increase in cancer incidence among the first group.

Cancer rates for those people increased by about 50 per cent in 1982 and remained higher than normal in 1983, but by 1985 the incidence rate had fallen below the historical average. "The pattern is quite striking and is unlike anything that had appeared previous to the accident," Hatch says.

The study included all types of cancers.

Hatch and co-workers rule out increased radioactivity as a cause for the increased cancer incidence because of a study they published last September in the *American Journal of Epidemiology* 132, 397-412; 1990).

There they compared cancer rates around Three Mile Island with recorded levels of radioactivity and found no significant correlation. The results were unsurprising because scientists already believed that the very low levels of radioactivity released by the accident were unlikely to have a measurable effect on cancer rates.

If the current findings are not just a statistical anomaly, Hatch believes the most reasonable explanation for them is that people living near the plant felt heightened stress in the months and years after the accident. This stress could have triggered increased cancer growth directly — a hypothesis that most researchers believe to be highly speculative — or it could have acted indirectly. Hal Morgenstern, an epidemiologist at the University of California, Los Angeles, notes that studies have shown stress causes people to be more likely to recognize and report various symptoms and to seek care for them.

In that case, the Three Mile Island accident could actually have done some people living close to the plant a favour by leading them to detect existing cancers sooner than they would have otherwise.

Robert Pool

Iceland sharpens its harpoons

London

A RESUMPTION of commercial whaling is in prospect after Iceland's chief delegate to the International Whaling Commission (IWC), Gudmundur Eriksson, said he will advise his government to leave the organization. Iceland's withdrawal from the IWC, which could take effect from 30 June 1992, would leave its whaling fleet free to hunt minke and fin whales in the central North Atlantic, irrespective of the wishes of the IWC.

The IWC imposed a ban on commercial whaling in 1986 when it was realised that the technique used by the commission to calculate catch quotas was seriously flawed: whale stocks could be depleted even by catches sanctioned by the IWC. Iceland grudgingly accepted this argument, but Norway and Japan, the other two main whaling nations, continued commercial hunting for another two seasons.

Iceland's strongest threat yet to leave the commission came in the closing minutes of an acrimonious IWC annual meeting, held in Reykjavik, Iceland, last week. Icelandic delegates were angered by what they saw as a deliberate attempt by the majority of anti-whaling nations within the IWC to delay the implementation of a "revised management procedure", devised by the IWC's scientific committee (see *Nature* 351, 259; 23 May 1991). This procedure (essentially a mathematical model based on whale population dynamics)

should allow the setting of commercial catch quotas that do not endanger whale populations — at least for some stocks of the relatively numerous minke. The IWC also refused to consider an Icelandic request to allow "interim" commercial catches of minke and fin whales over the coming year.

The IWC agreed to adopt the management procedure put forward by its scientists,

The resolution, passed by 18 votes to six, also asks the IWC's scientific committee to do more work to refine the procedure. The Icelandic delegation believes that the anti-whaling nations will use uncertainty over the precise size of whale stocks, and the positions of the boundaries between them, as an excuse to delay the resumption of commercial whaling within the IWC.

The Icelandic cabinet must now weigh the advantages of resuming commercial whaling outside the IWC against the threat of economic sanctions from the

United States. Under fisheries legislation, the US Administration can impose sanctions on countries endangering whale populations.

Norway is also now reconsidering its membership of the IWC. Japan, while expressing disappointment about the IWC's decision, has made no immediate threat to leave. The Japanese hope that, if the revised management procedure is implemented next year, they will be given IWC quotas to catch minke in Antarctica, where stocks are much larger than the North Atlantic populations that Iceland and

Norway wish to hunt.

But that hope may be optimistic. Next year's IWC meeting is in Glasgow, Scotland, and British public opinion is set firmly against whaling. The British government is expected to oppose a resumption of whaling on humane grounds, irrespective of the conservation issue, arguing that no humane method of killing whales exists. Many other IWC nations are expected to support this stand.

Peter Aldhous

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Japanese Embassy London: Greenpeace's memorial to whales killed since 1986.

even though this was seen as the first step towards the resumption of commercial whaling within the IWC, which most IWC members wish to prevent. But a resolution put forward by a hard-core group of nine anti-whaling nations — Australia, Finland, Germany, Oman, the Netherlands, the Seychelles, Switzerland, Britain and the United States — contains a number of caveats that will restrict the use of the procedure to set catch quotas.

Greens in disarray after committing a tactical blunder

London

THE threatened fragmentation of the IWC, and with it the return of commercial whaling, is a major disappointment for the environmentalist lobby, which had counted the IWC whaling ban among its successes.

But for environmentalist groups, the battle was lost not so much in last week's meeting of the full IWC, but rather during the two-week meeting of the IWC's scientific committee that preceded it. Once the IWC's scientists had chosen a single management procedure from the five they were considering, a clash between the anti-whaling IWC members and the whaling nations was inevitable (see *Nature* 351, 259; 1991).

The scientific committee, like its parent body, is a forum influenced by political hidden agendas, where scientists sympathetic to the environmentalist cause oppose those advising the governments of the whaling nations.

The anti-whaling members of the scientific committee had hoped the committee would be unable to choose between the five rival management procedures. Sidney Holt, scientific adviser to the Seychelles IWC delegation, says that all five procedures should have been put forward to the full IWC, with an explanation of each provided by the scientific committee. The choice of a single procedure "was a policy one, not a scientific one," he says.

But most committee members decided that the procedure developed by Justin Cooke, a British mathematician now working in Germany, was superior to the other four on offer — giving the best balance between protecting whale populations from depletion, maximizing catches and ensuring that catch quotas do not vary wildly from year to year.

Shocked by this development, a small group of anti-whaling scientists, ironically

including Cooke himself, objected to the selection of Cooke's procedure. If a single procedure was to be chosen, they said, it should be that developed by Bill de la Mare, an Australian who now works in the Netherlands. Among the five candidate management procedures, de la Mare's places the highest priority on the protection of whale stocks from depletion, and would delay the resumption of catches from heavily depleted populations for several decades. But the pleas of the antiwhaling scientists were to no avail, and Cooke's procedure was put forward to the full IWC.

The anti-whaling members of the scientific committee are seen to have committed a major tactical blunder, effectively being undermined by the mathematical talent of one of their own number. "They've shot themselves in both feet," said one informed observer in Reykjavik last week.

Peter Aldhous

COLLABORATION

Call for LINK reform

London

THE rules governing LINK, the largest UK government programme supporting industrial/academic collaborative research, should be overhauled, according to the government-appointed committee that oversees the programme.

The independent LINK steering group says that LINK should follow the practice of European Communities research programmes, and allow patents to be owned by whichever research group within a collaboration is responsible for an invention. At present, the Department of Trade and Industry, the government department most closely involved with LINK, prefers patents to be owned by the industrial partners in each LINK project. This is widely believed to have seriously hampered the programme. Bickering over the royalties to be paid to academic researchers in the event of any marketable invention has delayed many research projects.

The steering group's call for reform comes in response to a report from the management consultants Segal Quince Wicksteed (see *Nature* 351, 178; 16 May 1991), who were asked to examine why many LINK projects have been slow to get off the ground. The government had hoped that £420 million would be spent on LINK research between 1988 and 1993, with half coming from government and half from industry. But by November 1990, only 95 research projects, worth a mere £60 million, were actually under way.

The steering group has rejected several of the consultants' key recommendations, however. Many industrial companies claim that the government pays too little towards the research costs of the industrial partners in LINK projects. The government will pay only half of the total cost of each LINK project. Because academic researchers have their costs met in full, industrial collaborators typically have to cover most of their own research costs. The Segal Quince Wicksteed report suggested paying 35 per cent of companies' costs, to encourage more to take part.

The steering group, however, rejected this idea. Robert Malpas, steering group chairman, does not accept that the level of funding for industrial collaborators is a difficulty. The problem has simply been one of presentation, he says — the government has failed to convince companies of the benefits of collaborating with academic researchers in LINK projects.

The Segal Quince Wicksteed report also fuels the debate over the need for a cabinet minister responsible for science across the whole of government. There is no one person accountable to the cabinet for LINK, the report says. But until there is a minister for science and technology "there is no natural answer to this question."

Peter Aldhous

INDIA

Scientists will miss Gandhi

New Delhi

WITH the assassination of Rajiv Gandhi, Indian scientists have lost a major supporter. Gandhi, the former prime minister who was campaigning to regain that office when he was killed by a bomb, is described by Indian scientists as a politician with a technical mind who wanted to catapult India into the 21st century.

"With his death, Indian science has lost not just its champion but its very direction," said C. N. R. Rao, chairman of the science advisory council to Gandhi when he was prime minister.

Gandhi inherited a nation with a large scientific infrastructure and manpower, but he believed that Indian scientists were wasting time because their emphasis on self-reliance led them to reinvent the wheel. "He was a man in a hurry," said M. G. K. Menon, who was Gandhi's science adviser for five years. Gandhi, a professional engineer before entering politics, liberalized imports of foreign knowhow and technology, and coaxed Indian scientists to catch up with the West in an atmosphere of competition.

Gandhi took a personal interest in science, Rao said, which set him apart from his mother and predecessor as prime minister, Indira Gandhi, who also promoted science but let other people handle the details. When high-temperature superconductivity became a hot topic, Gandhi set up a commit-

tee of scientists with himself as chairman. Three years ago, after becoming impatient with US delays in selling supercomputers to India, Gandhi accepted Rao's proposal to set up a centre for the development of advanced computers. That centre has already delivered its first parallel-processing machine. Gandhi also encouraged the Bhabha Atomic Research Centre to go ahead with its cold fusion work despite criticism.

When India's ASLV rocket crashed in 1987, Gandhi called for a technical presentation by space commission chairman U. R. Rao. Gandhi "grilled [Rao] with questions only a technical man can ask," Menon said.

And after Gandhi introduced a computer policy that lifted import restrictions and spawned local computer companies, India's movement into the computer age noticeably accelerated, said N. Seshagiri, chief of the National Informatics Centre. Seshagiri attributes the commissioning of the centre's computer network, NICNET, to Gandhi, who took a personal interest in it.

Gandhi considered the labyrinthine Indian bureaucracy as the main obstacle to scientific progress, Rao said. Although out of power after the 1989 election, Gandhi was working on a new blueprint for science and technology and a scheme for restructuring the science establishment to free it from the grip of that bureaucracy. **K. S. Jayaraman**

INTERNATIONAL COLLABORATION

India and Australia plan ties

New Delhi

A GROWTH in scientific cooperation between India and Australia is expected with the signing of an agreement between the Indian Council of Scientific and Industrial Research (CSIR) and Australia's Department of Industry, Technology and Commerce (DITAC).

Until recently, scientific cooperation between the two countries was at the level of individual scientists. Now, for the first time, CSIR, India's principal scientific agency, will come into close contact with its Australian counterpart, the Commonwealth Scientific and Industrial Research Organization (CSIRO).

Michael Pitman, the DITAC science adviser who recently led a ten-member delegation to India, said there is "substantial scope for increased collaboration involving a wide range of institutions in both countries". The agreement was signed at the conclusion of a ten-day tour by the Australian delegation, which visited more than 20 of India's scientific institutions in order to identify areas of mutual interest.

Under the agreement, India and Australia will exchange scientists, conduct joint workshops and conferences, and carry out joint

research projects in certain areas. Marine science and new materials are two that have been given priority. Others are catalysis, molecular biology, food technology, information science and atmospheric physics.

Specific projects for joint research have not yet been worked out, said Barry Filshie of CSIRO's international division, but he offered several possibilities on the basis of discussions so far. The two countries might study new silicon materials for converting sunlight into electricity or the emission of greenhouse gases from paddy fields and cattle sheds. There could also be joint work on scaling up a commercially promising Indian process for converting natural gas into kerosene and diesel fuel. Both India and Australia have surplus natural gas, so "the technology could be of mutual interest," he said.

Indian scientists also expect to try out some unique Australian fodder species for reclaiming saline land, and, according to K. N. Johry of CSIR, Australian dairy scientists are keen on an Indian process for making vegetarian cheese.

Filshie said that DITAC has allocated A\$110,000 for collaborative projects with India. **K. S. Jayaraman**

Rebuilding takes a downturn

Munich

WHEN Bonn agreed two weeks ago to earmark a further DM1,760 million (about \$1,000 million) to help rebuild universities in eastern Germany, it seemed on the surface to be an encouraging step. But a number of signs, including last-minute reductions in the amount of the payments, indicate that things may not be as rosy as they seem and that the aid might run out just when it is needed most.

The latest segment of the programme, which was approved by Bonn and the *Länder* (states) on 24 May, will help the former East German universities to rebuild departments — such as law, economics, social sciences and the humanities — considered tainted with Communist ideology. The new programme will also pay university salaries for as many as 2,000 researchers from the former East German Academy of Science and two other academies, and will create 650 five-year positions for young researchers and graduate students.

The programme was originally expected to provide DM3,000 million (about \$1,765 million) over five years, but it had to be reduced by 40 per cent when the *Länder* in

RESEARCH COLLABORATION

Charities cohabit in Cambridge

London

NEXT week sees the opening of a new £5 million institute for developmental biology at the University of Cambridge, the first major British research institute to be funded jointly by two of Britain's leading medical research charities.

The new institute will initially house some 12 research groups, under the direction of Cambridge developmental biologist John Gurdon. The goal is for Cambridge to become an internationally competitive centre for UK developmental biology, bringing together work on the mouse, *Drosophila*, *Xenopus* and other model organisms.

The institute's £5 million capital cost and the £2–£3 million annual budget are being provided by the Wellcome Trust, which will pay two-thirds, and the Cancer Research Campaign (CRC). Michael Morgan from Wellcome says the collaboration was possible because the two charities have complementary research interests. The fact that Wellcome has a policy of not supporting cancer research simplified the division of responsibility for funding between the trust and the CRC, which was potentially a tricky problem.

The two charities decided to fund the institute on the basis of its scientific merit, Morgan says, but a secondary consideration was a desire to halt the 'brain drain' of British developmental biologists moving abroad.

Peter Aldhous

western Germany pulled out. Now, the federal government will pay for three-fourths of the programme, and the eastern *Länder* will provide the rest.

The reduction will make a "significant difference", says Peter Gutjahr-Löser, chancellor of the University of Leipzig, because the eastern German universities will not be able to compete effectively with western German universities for professors.

Eastern universities do not at present have the money to pay their faculty more than 60 per cent of average western salaries. In its original form, the new programme would have paid salaries at western rates for more than 200 professors or assistant professors at Leipzig alone, but now that has been reduced to fewer than 60.

Even worse for the eastern universities, the programme will now pay for only two years to resettle academy researchers at universities, instead of the original five. This defeats the purpose of the programme, says Jochen Stoehr, head of the research division of the science ministry in Berlin.

The financially strapped eastern universities will not take on academy people, Stoehr says, because they will have to pay them out of their own funds in just two years. Five years would have allowed time for more long-term positions to open through retirement.

The Federal Ministry for Research and Technology has promised that the programme will be reviewed in two years and possibly extended, but Stoehr says that by then it will be too late.

At the heart of the funding problem is the fact that the west is approaching the limit of its generosity. In February, the western *Länder* agreed to transfer DM4,800 million in income from the 'turnover tax' on business transactions to eastern Germany. Altogether, the western states have poured DM80,000 million into various funds to help eastern Germany, with the result that western universities are now facing cuts.

So, in February, the western *Länder* said that they had done all they can for rebuilding eastern Germany, and that it would be up to Bonn to do the rest.

But the federal government will not be able to go much further in improving conditions at eastern German universities, either. As the negotiations on the 1992 German budget draw to a close, it seems very unlikely that the Research Ministry will get any increase in its projected budget of DM8,400 million.

The chairman of the science advisory council Wissenschaftsrat, Dieter Simon, expresses disappointment over the lack of solidarity showed by the west for the east. "They don't realize that the old West Germany has gone under," and that the annual game of trying to get more money from taxpayers or Bonn each year has ended, he says.

"The new game is about sharing what we've got." Wissenschaftsrat had recommended that the programme be funded at the original figure of DM3,000 million.

Gutjahr-Löser at Leipzig argues that it is shortsighted of the west not to devote more support to eastern German education and science. The number of students in the whole of Germany continues to increase, he notes, and the eastern universities, which have traditionally taught many fewer students than the western universities, will have to shoulder much of the added burden. But the eastern schools will be hard-pressed to do this if they cannot attract good faculty, and this in turn will increase the pressure on already overcrowded universities in the west.

Underlining the seriousness of the situation, the president of the German university rectors' conference, Hans-Uwe Erichsen, said in April that unless something is done soon, a "collapse in the German university system" can hardly be avoided.

Some observers have criticized the eastern *Länder* for not refusing to accept the trimmed-down version of the new programme. Simon disagrees. It would have been "suicidal" not to accept it, he says, because "they need all the help they can get."

Steven Dickman

EARTH SCIENCES

Scientists watching Japanese volcano

Tokyo

ALL eyes in Japan are on an erupting volcano in the southern island of Kyushu which killed 15,000 people the last time it burst into life 200 years ago.

Mount Unzen, near the town of Shimabara in Nagasaki Prefecture, began smouldering last November and then became comparatively quiet a few weeks later. But intense seismic activity resumed earlier this year and by the middle of last month a cracked dome of viscous lava emerged in the volcano's crater. A few days later, the volcano burst into violent eruption with flows of ash and volcanic gas cascading down the mountainside to within a few hundred metres of residential areas.

Then on Monday (3 June), the volcano spewed a pyroclastic flow — a mixture of gas and rocks — into a nearby residential area, which had earlier been evacuated. Initial reports were that 20 people were injured and 28 missing. Many of the missing were apparently researchers and journalists who had not left the area.

When the volcano last erupted in 1792, it was not pyroclastic flows that killed most people. Rather, earthquakes associated with the eruption set off a landslide on a nearby mountain, triggering a tidal wave that swept away thousands of people. Scientists observing the mountain are keeping a close eye on the locus of earthquakes in case the same happens again.

David Swinbanks

US looks to German model

Bonn

IN a search for a solution to the estimated 32 million people lacking medical coverage in the United States, supporters of a major overhaul of the US health care delivery system are shifting their sights away from the government-run Canadian system towards the more market-orientated German system.

US health policy analysts and politicians have begun to visit Germany to study how the 108-year-old German scheme, first established by Bismarck, provides high-quality medical care to all citizens while avoiding medical inflation. Members of the US House of Representatives Ways and Means Committee, for instance, are considering a trip to examine Germany as a possible model for reform.

At the same time, numerous reports extolling the virtues of the German system have begun to appear in US academic journals and the press. Observers from across the US ideological spectrum say that Germany's example may be more pertinent than Canada's or Britain's because of US anxieties over government control and tax financing.

The quest for a new US health care strategy is particularly pressing because public sentiment for reform of the health care delivery system is at unprecedented levels. A 1990 Conference Board poll found that Americans are more concerned about medical costs than about crime, pollution, AIDS, unemployment or homelessness.

US budget director Richard Darman startled many when he told Congress in April that US corporations will pay more for health insurance this year than they make in total profits. Not surprisingly, many large businesses, including Chrysler Corporation, have joined labour unions in calling for major reforms.

The missing catalyst, many observers say, is a significant show of support from the Bush Administration.

Meanwhile, US economists fret about the growing share health care takes from the gross national product (GNP). In the past decade, health spending outpaced overall economic growth by 33 per cent, rising from 9.3 per cent of GNP in 1980 to 12.2 per cent last year, or \$756,000 million. Some economists fear that, left uncorrected, health costs could consume 25 per cent of the US economy by 2010. Spending on health care stood at \$2,354 per capita in 1989.

But how should a US national health care system be structured? The years of debate over importing the more-or-less socialist Canadian system southwards were just a "silly diversion", says Princeton health economist Uwe Reinhardt. Instead, he predicts, the United States "will eventually stumble" towards a German-like system of controlled markets, but retaining a strong emphasis on private physicians and employer-financed health insurance.

Germany certainly avoids many of the problems that dog the US health care system. Health care's share of the former West Germany's GNP has hovered around 8.5 per cent since the mid-1970s, and even declined from a peak of 8.9 per cent in 1988 to 8.2 per cent in 1989. At DM1,838 (\$1,232) per capita, Germany spends half what the United States does, but has lower infant mortality (tenth versus twenty-fourth among industrialized nations, according to the World Health Organization) and matches average US life expectancy (75 years).

Germany has achieved cost control in the face of significant demographic odds. Fifteen per cent of the population is over 65, compared with 12 per cent in the United States. The doctor-to-patient ratio is the world's highest, and some 20 per cent higher than in the United States. Germans average 11 visits to a physician a year, compared with four in the United States. Hospitalization rates are 44 per cent higher, hospital stays are twice as long and the number of hospital beds per capita is double that in the United States.

What is attractive to US policy-makers is the federal republic's blend of structural elements familiar to US patients — privately administered insurance plans, nearly free choice of physicians and hospitals and virtually immediate access to all services, including high-technology procedures. Both systems are funded by employer contributions, although German workers pay 50 per cent of the premium.

Germany's main strategy is to set limits on overall spending by parliamentary edict, leaving nearly all the operational details to hospitals, physicians and insurers. This "steering" role, says Professor Michael Arnold of the University of Tübingen, insulates the government from the pressures applied to bodies that act as direct payers, such as the Canadian provinces and the US Medicare and Medicaid systems.

Much of the new attention on Germany is focused on the formal price-fixing mechanisms for physician fees and hospital rates. US physicians are particularly intrigued by the negotiations conducted by insurers and regional "doctors' payment organizations", which have legal authority to bargain over fee increases. Such collusion over fees would be illegal under US anti-monopoly rules, although Senate majority leader George Mitchell (Democrat, Maine) is said to be drawing up a proposal that would exempt health care providers.

Since 1986, rises in German physician fees have been directly tied to the average worker's wage fluctuations, while US fees have risen without controls. "To provide first-dollar coverage as we do, you need a fee system for physicians and binding negotiations," says J.-Matthias Graf von der Schulenburg, an economist at the University of Hannover. "If you don't, you'll always

have doctors charging more."

Although German physicians earn significantly less than US colleagues, due in part to the fee limits, they have retained a relatively high economic position in society. The average pretax income for German physicians is DM160,000 (\$107,200), compared with \$155,800 in the United States. German physicians earn four times the average worker's wage — down from six times in 1983 — while US physicians make 6.7 times more than the average worker.

Because of their potential impact on income, expenditure limits were fought fiercely by the American Medical Association (AMA) when proposed in Congress two years ago, but organized medicine now seems more receptive. The AMA has campaigned vigorously against any movement towards a Canadian- or British-style system.

The economic pressure of technological advances in medicine is as evident in Germany as elsewhere, although strict government control over spending on expensive equipment has improved efficiency, German economists say. Nevertheless, physicians exploit the payment system for additional fees by practising what some call "machine medicine".

As in the United States, rises in payments for technical procedures have outpaced those for cognitive services, providing physicians with an incentive to run more tests. As a result, the typical German generalist has an array of money-generating devices in the office that would impress US physicians. Most have ultrasound scanners, sophisticated blood analysers and X-ray equipment.

The German system also achieves significant efficiencies by keeping administrative costs low. For instance, German physicians maintain only one insurance form per patient per quarter, whereas each US physician visit generates an average of 11 pieces of paper.

As bright as the German system may appear in the United States, significant problems lie ahead. "We have too many doctors to employ at a reasonable price," says Peter Rosenberg, a federal health economist in Germany, where an estimated 6,400 physicians are unemployed. Germany graduates as many physicians each year as does the United States and there are constitutional protections guaranteeing career choice that inhibit efforts to limit medical school admissions.

Looming even larger are the possible effects of unification. The 1990 treaty eliminating the frontier essentially called for a wholesale replacement of the former East German socialized system with the West's entrepreneurial one. German officials have estimated it will take as much as DM105,000 million (\$70,000 million) to remake the east according to western standards.

Joe R. Neel

Joe R. Neel, a reporter in the Washington bureau of *Physician's Weekly*, was recently in Germany.

Biology's variable logic

SIR — Is it not time to reconsider seriously the concept that applied mathematics (physics style) is not relevant to the training of biologists? Or should we continue to obey Kantor's law of the conservation of ignorance, namely that an incorrect concept accepted, taught and used for many years by many intelligent people is hard to change; and the less the concept is understood, the harder it is to change?

Thirty years ago, the management of the chemical company I worked for, as an applied mathematician, assigned me to the Animal Science Group, which was experimentally comparing three types of synthetic substitutes for dietary methionine for improving the growth of chickens from day-old to market age. The group was not particularly happy about this, as they already had a statistician well versed in Fisher's approach to biological experimentation, and it was already known that mathematics was not relevant to biology. I saw Brody's *Bioenergetics and Growth* on their desks, but they said they didn't use it.

My assignment was to get some idea why there was so much confusion between the experimental results of our research group and the various academic groups that were engaged to test the products under conditions and with chickens as closely like ours as possible. I found that the basal diets used were somewhat different because the local ingredients varied, as was expected; the methods of incorporating the additives in the basal differed; the durations of the experiments varied so that there were differences in the mean weight gains for chicks; and the statistical treatment of the data differed.

However, my study, beginning with the historical background of the experimental techniques used in such nutritional studies, seemed to make it clear that a major cause of the confusion was the lack of a commonly known and not too difficult to understand theory of how animals grow, depending on the composition of the freely fed diets.

My colleagues were aware of my work; a few understood but most did not and rejected it anyway in favour of what the majority were thinking and doing. I later published a book¹ on the approach I was taking.

Recently, L. M. Potter *et al.*² have presented a study of the same kind of confusion and controversy surrounding the same problem I had to deal with in the 1960s. The state of affairs then and now is typified by the following quotation from their paper.

Although a variety of studies exist on the methionine active compounds available to the feed industry, results show apparent variations because experimental designs and basal diets differ among studies and therefore it is risky and possibly inaccurate to draw conclusions from the literature without careful observation. Periods within a study can even alter results.

Obviously, selection of particular studies can influence the position one establishes.

Is it not a great shame that so many young people have been trained over the past 50 years to accept such work as scientific and worthy of publication, and that the peer-review process can only fail due to the insidious character of Kantor's law? Can we be sure the same situation is not also present in other fast-growing fields of biology?

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Missing the point

SIR — The term 'computational biology' is very new to science, even newer than the term 'computational chemistry'. As a field distinct from both theoretical biology and mathematical biology, computational biology can be simply defined as the application of computational methods to the study of biological systems. As such, the scope of computational biology is as broad as the range of computational methods at our disposal and the extent of biological problems intriguing us. Examples of applications of computational biology include modelling and simulation of dynamic processes at each hierarchic level from biomolecules up to societies of organisms, as well as modelling the structure and organization of each of these levels and how that relates to function. Computational biology problems arise in biochemistry and molecular biology, physiology, ecology, evolutionary biology and so on.

I am concerned that some members of the scientific community may have too narrow a view of this field and that others may adopt this narrow view, to the detriment of the field. For example, two recent symposia in the United States^{1,2} which were presented as symposia on 'computational biology' actually focused on a subset of computational biology, that of research in protein and nucleic acid sequence comparison and biomolecular structure. The use of the term 'computational biology' in the scientific literature has also reflected the same limited scope³. Baylor College of Medicine and Rice University have established a Center for Computational Biology, but at least two-thirds of the faculty are specialists in biomolecular structure.

This narrow use of 'computational biology' is unfortunate, because the scope of this field is actually much wider. In the same sense that applications of computational chemistry are not restricted to quantum mechanics and molecular dynamics but also

include chemical kinetics and prediction of organic reactions, applications of computational biology include everything from protein structure to predator-prey population dynamics to systems physiology.

While the narrow use of 'computational biology' may reflect greater attention to biomolecular structure as a result of the Human Genome Project, this use may result in insufficient appreciation for other areas of biology that are being studied by computational means. Recognition of the success of and future potential for computational biology in dealing with questions of human health and world ecology will begin by understanding its widespread applicability.

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Frosty foods

SIR — The notes on some innovations and inventions, "refrigeration (1895)", "frozen foods (first sales 1930)" and "food preservatives (1873)" in Jesse H. Ausubel's article (*Nature* 350, 649; 1991) require modification.

Refrigeration became available on an industrial scale from the development, by D. Boyle, of the compression-expansion refrigerator using ammonia as the heat transfer fluid in 1872.

Frozen food (meat) was first imported into the United Kingdom (from Australia) in 1880 and by 1910 imports had risen to more than 500,000 tons a year.

A novel food preservative may have been developed in 1873 but the general principle exemplified by the use of salt, sulphur and sulphur dioxide had been used since pre-Roman times.

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Source please

SIR — Although the authors of "Bayesian reasoning in science" (*Nature* 350, 371; 1991) mentioned Bayes's original work, entitled "An essay towards solving a problem in the doctrine of chances", they did not reference it. His original work, although rather lengthy, is available quite easily in *The Philosophical Transactions of the Royal Society of London*, 53, 370–418 (1763). A correct reference should always be given to source material.

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Politics of electricity production

Terence Price

The nuclear industry is seriously addressing the issues which cause public concern. Despite its still controversial image, a wave of nuclear construction is likely to begin soon after the year 2000.

ALMOST everywhere, the antinuclear lobby remains entrenched and vigorous. Italy has dismantled its nuclear programme at popular insistence. Austria complains to Czechoslovakia about its nuclear power stations. There is a moratorium in Switzerland on new reactor construction. Denmark has a non-nuclear energy policy. The United States is riven by arguments over nuclear licensing and waste disposal. Britain has paused for thought until 1994.

At the same time nuclear power makes about 17 per cent of the world's electricity — as much as came from all sources in 1957. The United States leads in the number of operating power reactors — currently 112. Ten of the 25 countries with operating reactors make more than a third of their electricity this way. The figure for France is 74 per cent. French exports keep the Italian and Swiss grids in balance. Austria draws on electricity from Czech nuclear stations, just as Denmark does from Swedish electricity, which is 50 per cent nuclear. Nuclear waste technology poses no great difficulties, politics apart.

This disparity between perception and reality must be dealt with before nuclear power can make its proper contribution in a world whose population will be 60 per cent larger by 2025. Even allowing for conservation and efficiency, 50 per cent more energy will be needed by then. It cannot come on that scale from the renewables. Only coal, gas, oil, hydro and nuclear power produce really big packets of energy. The first three have the greenhouse question mark hanging over them, while hydro resources are limited. Reductions in the emissions of carbon dioxide, already promised by Europe and Japan, would be impossible without a full nuclear contribution.

There is scope for at least a doubling of nuclear electricity over the next 30 years. But today the nuclear construction industry's order books are almost empty. The known prospects for new orders can be counted on one's fingers, a trivial addition to the 429 power reactors already completed, and the 83 still under construction.

Starting over

Can the industry be got moving again, and in time? The problems are almost entirely political: before the politicians will act, the public's concerns about economics, safety, waste disposal and nuclear proliferation must first be allayed. Meanwhile there must be no more serious accidents. On all these issues the nuclear industry has a far better case than

it did before Three Mile Island and Chernobyl engendered a new realization that its future depends on the elimination of weakness at every level, everywhere.

Few issues have been more needlessly confused than nuclear economics. The truth is simple. If stations of standardized and proved design are built, under a regulatory regime that permits construction in a reasonable time of about 5 years, then they should be economic except near sources of very cheap coal. Conversely, stations with 'one-off' designs, requiring detailed development, start with a fundamental disadvantage. If in addition regulatory arrangements require a completed station to lie idle, possibly for years, with financing costs mounting while objectors challenge its licensing in the courts, its chances of competing economically virtually disappear.

Standardization has been the key to the success of Electricité de France; elsewhere it has not yet got far. The German 'convoy' programme shrank to only three reactors. The United States similarly has only a handful of identical stations. The three reactors at Palo Verde, in Arizona, show the benefits that have been missed: unit 2 was 5 per cent cheaper than unit 1, and the start-up time was cut from 56 to 35 weeks.

Britain in the 1950s and 1960s took the opposite route of promoting competition, thereby committing the industry to sorting out the teething, training and logistical problems of several different reactor types. Furthermore, when in the 1980s the industry belatedly switched from gas-cooling to water-cooling, the domestic politics of privatization limited the programme to one reactor at Sizewell, thus missing the opportunity of spreading the design and start-up costs over a batch of four reactors, as originally intended. The result is that Sizewell B will produce electricity 50 per cent more expensively than the similar French stations just across the English Channel. The British public has been given the false impression that nuclear power is inherently uneconomical.

Further confusion arose from the Swedish government's assertions, backed by legislation in 1987, that the country's 12 nuclear stations — making half of its electricity — could be phased out and replaced by other forms of generation by the year 2010, without economic penalty. Eventually, harsh reality forced a change of policy. The decision to close the first two reactors, in 1995 and 1996, had in any case run foul of the Swedish parliament's policy, adopted in

1988, constraining coal and hydrocarbon use in power stations for 'greenhouse' reasons. The government this year abandoned the phasing-out of the two reactors.

The public fears radiation. The message needs to be spread that knowledge of its effects on the human body is by now very detailed. It comes from many sources: medical exposure; occupational exposure; occupational health follow-up studies of the atomic-energy work-force; mining experience before the hazards of the naturally occurring radioactive gas radon were fully appreciated; animal experiments; Hiroshima and Nagasaki; and now Chernobyl. Such information is kept under review by the International Commission on Radiological Protection (ICRP), and by UNSCEAR, the United Nations Scientific Committee on the Effects of Atomic Radiation. The ICRP made new and more stringent recommendations as recently as November 1990.

Doubts

Where doubts remain, investigations continue, as over the issue of childhood leukaemia. Around the UK Sellafield reprocessing plant the study by Martin Gardner and colleagues (*Brit. med. J.* **300**, 423–429; 1990) seems to have pinpointed a small but statistically significant incidence among the population, many of whom came from elsewhere when the plant was commissioned. But Leo Kinlen has shown that similar small increases can also be found in new towns built since the Second World War, with no nuclear involvement (see *Nature* **342**, 213; 1989). It is not yet known whether the cause is a virus brought to a district by immigrant workers, or radiation; but the medical profession is in pursuit, not least for reasons of self-protection.

The nuclear industry is kept on its toes both by its critics and by the lessons of experience. Three months after Three Mile Island a new Institute of Nuclear Power Operations (INPO) was set up in the United States to promote operating excellence. After Chernobyl, as the deficiencies in Soviet safety practices became clear, INPO helped to create a new World Association of Nuclear Operators (WANO); the inaugural meeting in May 1989 was held in Moscow. Through WANO, plus many country-to-country assistance agreements and the advisory programmes of the International Atomic Energy Agency (IAEA) in Vienna, there are now regular peer-group safety and reliability reviews. Where lapses are uncovered, such as the notorious case at Peach Bottom in March

1987 in the United States (operators were caught sleeping on duty), personal and corporate punishment is well deserved.

As the United States is the largest user of nuclear energy, and home of many environmental organizations, what happens there is of particular significance. It is therefore important to make allowance for the uniquely cumbersome US licensing and regulatory system, which has required public inquiries both before construction is started and after a reactor is complete. The result is that some reactors have remained idle for years, accumulating financing charges which, under another US law, could not be passed on to the consumer until the stations satisfied the test of being 'used and useful'.

New regulations

In 1989 the US Nuclear Regulatory Commission recognized the difficulty by publishing new regulations that went a long way towards meeting the industry's concerns. The aim is to complete essentially all licensing formalities at the outset, so that only construction checks are needed subsequently. In addition, there are moves towards standardization of a new family of reactors, which should permit licensing by type, as with aircraft. Although there is vigorous opposition, the probability is that necessity will soon force the United States to create a new licensing regime acceptable not only to Congress but also to the electricity industry.

Everywhere there is public anxiety about the disposal of radioactive waste. Yet 'geological' disposal in deep repositories is a fairly straightforward technology. The volume of the most highly active material is small, once it has been concentrated in a chemical reprocessing plant: the first 30 years of Britain's nuclear programme produced only 1,100 cubic metres of such waste. There is no difficulty in identifying suitable deep sites that are geologically stable and where groundwater movement is minimal. The material can be vitrified in glass blocks, which can then be surrounded by barriers to give further protection. By including concrete as one of the barriers, thus creating alkaline storage conditions, the movement of most of the 30 or so fission product species important to human health can be inhibited.

Once again the real problem is political: the exploratory drilling used to locate underground storage sites alarms local inhabitants and their representatives. But eventually governments must face up to the task of explaining to the electorate that there is really nothing to fear. Sweden has moved faster than most countries, and has already built two major facilities.

The reason why this relatively simple problem was not dealt with earlier is that, in a technical sense, there was no real hurry. The highly active fission products coming out of a reactor initially produce too much heat for safe underground storage, first needing to be left to cool in a surface storage facility for around 50 years. Surface storage has been

proceeding for three decades, in most cases with few problems, either technical or political. Unfortunately the rushed wartime development of nuclear weapons in the United States led to the dumping of radioactive materials at a number of sites with few precautions, notably at Hanford. The publicity over cleaning up Hanford has rubbed off adversely onto civil nuclear power. At the Soviet Union's Chelyabinsk-40 weapons production site the situation is even worse.

The nuclear industry needs to break the association in the public mind between the well-supervised planning of civil nuclear power and such legacies of military haste. Part of the public's disquiet about nuclear electricity must be a matter of association: the same word — nuclear — describes the most lethal form of weaponry. Moreover, some of the technology is common to both peaceful and war-like applications. It is the pragmatic aim of almost every government to retain the benefits of this new form of energy while erecting the strongest possible political barriers against weapons proliferation. The Nuclear Non-Proliferation Treaty (NPT) of 1968 has been one of the great political successes, with 141 out of the United Nations' 159 countries as signatories. States without nuclear weapons have thereby agreed to permit inspection of their nuclear facilities by the IAEA. The resulting formalities are part of the daily life of every manager of nuclear fuel.

A few 'threshold states' have resisted falling into line. India and Pakistan both have the technical potential for making weapons in the near future; India indeed exploded a crude 'peaceful' device as long ago as 1974. South Africa is still considering whether to place its nuclear plant and equipment under international control. Argentina and Brazil signed on 28 November 1990 an agreement arranging for mutual inspection of nuclear installations under the aegis of the IAEA. Israel is believed to have between 50 and 150 nuclear warheads. North Korea acceded to the NPT in 1985, but there is still no agreement on 'safeguards' inspection.

Backing up the NPT is the Nuclear Suppliers Group (the 'London club'), formed in 1975 to exert political pressure to prevent any leakage of nuclear materials which might assist a clandestine weapons programme. It now includes 26 countries, whose governments apply what pressures they can. The United States suspended new economic aid to Pakistan, worth \$600 million a year, on 1 October 1990, because the US President was unable to certify to Congress that Pakistan did not have a nuclear weapons programme. Germany is reluctant to help Iran to finish the two reactors at Bushehr. The Soviet Union is threatening to withdraw assistance from North Korea. Carrots are used as well as sticks: following the Brazil-Argentina accord, both the United States and Canada signalled their willingness to resume trade in nuclear materials.

Nuclear objectors can still point to prolife-

ration as a potential problem; but it is becoming increasingly difficult to argue that, by not building a reactor in Europe or the United States, the Middle East or the Indian subcontinent can be kept on the path of nuclear rectitude. A quarter of a century of quiet diplomacy has largely dealt with this once persuasive anti-nuclear argument.

Despite this progress, there is still no simple way in which the nuclear industry can influence public opinion in its favour. The motivations are too diverse, and there is no leader with whom to bargain. Nevertheless, in the next 10 years there should be a perceptible shift in the balance of the debate between those who wish to exploit the benefits of nuclear energy, and those who think otherwise. The common element in various nuclear anxieties is that the circumstances of the past 10 years have played into the objectors' hands, giving them a status that is only partly deserved. They have been able to point to Three Mile Island and Chernobyl; to poor economics when nuclear programmes are badly conceived and managed; to Sweden's phase-out policy; and so on.

The objectors' own main error has been to disregard the ability of others to learn. Nuclear power can be fully economic almost everywhere, provided standardized designs are used. In the most important nuclear market, the United States, expensive regulatory delays are now being addressed. Sweden has abandoned the first steps of her phase-out policy. Three Mile Island and Chernobyl have dramatically changed attitudes to safety. There is a drive everywhere to deal with the problem of nuclear waste. Weapons proliferation seems less threatening than it did when President Kennedy looked forward with apprehension to a world containing perhaps 25 nuclear weapons. The greenhouse argument is changing attitudes.

Climate for change

There are thus grounds for thinking that before the year 2000 a new context will have been created — so that, allowing for the normal delays for planning, financing, licensing and construction, a new wave of nuclear reactors should begin to appear in 15 years' time. I expect installed nuclear capacity to at least double by 2020, with nuclear electricity supplying one-tenth of the world's expanded energy needs. That is far from being a total replacement for other energies; but it is likely to be more than from hydro power, far more than from the new 'renewables', and one-quarter or one-third of the forecast contribution from coal. That is far from negligible, particularly for an energy source that did not exist half a century ago. With this as the prospect, it is surely time for the existence of nuclear power to be a matter for self-congratulation, not breast-beating. □

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Growing up with your software

Stephen Wolfram, the designer of *Mathematica*, the computationalist's software package, hopes to get back to physics soon. But will the bug let him go free?

STEPHEN Wolfram, the *enfant terrible* turned software salesman, is looking forward to a brief sabbatical, which he plans to use for writing a book on some aspect of complex systems. He reckons that he will be able adequately to keep in touch with his business, Wolfram Research Inc., which is based in Illinois. Even if he should decide to spend his time off in California, he reckons that electronic mail should serve as well from there as it does now from wherever else he is.

Wolfram is, of course, a phenomenon. I first heard about him more than a decade ago from W. H. ("Willie") Fowler, who was boasting over lunch one day that Caltech (otherwise the California Institute of Technology) had just acquired from Britain a prodigy who had taken his PhD in just over a year. The truth is even more startling. Wolfram had abandoned his undergraduate physics course at Oxford after a year and a bit, and signed up instead as a graduate student at Pasadena. When he graduated in 1988, he had just turned twenty. He had been hoping to become the only teenager of his acquaintance with a PhD, but he was beaten by a few months.

That was in 1979. Last week, Wolfram was in London briefly after touching base in twelve European countries in fourteen days. He was off to Cambridge later in the evening, and then to the Soviet Union. The objective? To do propaganda for *Mathematica*, his own software package whose power, he claims, now extends beyond that of being a computer program capable of handling symbolic algebra to being a computer language in its own right. It can also produce the square root of π to, say, 40 decimal places as quickly as if it were retrieving a predefined variable from immediate memory.

Wolfram has always been a computer buff. I first met him a few years after I first heard about him, when he had been exiled (if Princeton will forgive the term) to the Institute of Advanced Study. What had happened was that Wolfram had developed a computer program for calculating Feynmann integrals and had then broken the academic rules by founding a corporation to sell the product to his fellow academics.

His obsession at Princeton was the cellular automaton — von Neumann's conceptual device for representing a complex dynamical system by an array of simple microscopic elements (usually two-valued switches that are either ON or OFF, whose evolution in the course of time is determined by simple rules determining their interaction with other elements in the array. The serious objective was that of solving unsolved problems in fluid

dynamics, but Wolfram had built an impressive collection of sea animal shells whose markings seemed evocative of the orderly patterns that could be induced to emerge from the chaotic behaviour of an array of cellular automata.

His toy was a machine the like of which I had not then seen elsewhere — a desktop computer with 1 megabyte of immediate memory. The need was plain — 1 megabyte would be just enough to store a single image of high-definition screen. With the hardware ahead of the software, he had written the operating system himself. There were some hundreds of megabytes on hard disks in a separate box on which successive images would be stored. Sensing my envy of the toy, Wolfram advised me to buy stock in its manufacturer, now well-known as Sun Microsystems. As the world now knows, it was good advice. Wolfram is not to blame that I did not take it.

Now, at what must be all of thirty-three, Wolfram has not changed much. He was stocky ten years ago, and may be a little more so now. His hair has receded a little, making his forehead seem even broader, but is no tidier. He jeered politely about the restaurant, where neckties are required, but was not wearing sneakers. He remains buoyant with enthusiasm, and for *Mathematica* in particular, although he is also proud of the all electronic journal *Complex systems*, which seems to have been the first to give its mailing address as a Bitnet number and its "Instructions to authors" as a series of instructions for the typesetting language $T_E X$.

Mathematica is meant as a way of doing mathematics by machine that has its roots in Wolfram's automation of cellular automata nearly a decade ago. It seems also to be a product of Wolfram's own belief that, at least if you are he, the only way to come by a satisfactory program is to design and build it yourself. One might feel the same about one's motor-car, but it needs very little forethought to conclude that a person who insisted on building his own machines would have very little time left for using them. That is why Wolfram has become a business.

By his own account, it is also a successful business. "I have made a lot of money." There are no outside shareholders — only himself and his employees (who number about sixty). He is fiercely proud of the structure of his business, saying that he learned from his earlier venture that there is no point hiring a bunch of accountants and administrators to run the shell of a company within which the innovator will be coddled. Instead, you must begin by doing everything yourself,

handing over parcels of work that can be successfully delegated.

But can a person so closely identified personally with a computer program delegate to others responsibility for its future? Wolfram is forgivably ambiguous on this point. Users are always suggesting elaboration (although not as often as he had expected). He can, he says, leave it to others whom he trusts to add extra features to the system, but is less willing to delegate responsibility for new developments that are likely to affect the design of the system. And the crucial elements of the design are those that touch the interface between the program and its users, which determine how conveniently it can be used. One guesses that Wolfram will be worrying about the shape of *Mathematica* for, to a first approximation, the rest of time.

The users of *Mathematica* (more than 100,000 of them are registered, which means that they have paid for their copies) are scattered about the world. About a quarter of them are engineers, which is not surprising. For are not engineers the heaviest users of mathematics and at the same time the users least interested in the mathematics as such? But Wolfram is also delighted that the program has turned out to be a splendid way of generating educational materials as in the teaching of elementary calculus. His ambition for it seems to be that of a high-level shell that will support, as they say in the trade, any useful application its users may wish for.

Where does this leave Wolfram as a scientist? The next step is to be the book on complex systems. He also believes the epistemology of quantum mechanics to be ripe for sorting out. The grand problems of the Universe are by comparison less interesting. But, of course, there is also always the business of what will happen to computers and their capabilities. He is not interested in having *Mathematica* made capable of proving Euclid's theorems, but believes it has the potential to do thinking of a kind.

Wolfram carries *Mathematica* about with him, on a portable Compaq with 10 megabytes of immediate memory and a hard disk. He startles the waiters in the necktie restaurant by opening it on the linen tablecloth to show its tricks. "Look, here are the first forty digits of the square root of π , now ask for something else." So we tried a few integrals and solved the odd quadratic equation. He set up a cellular automaton, but it turned out to be a boring one — no chaos and so no order out of chaos. The toy now is not the machine, but the program. It will be interesting to see whether he can leave it alone during his sabbatical.

John Maddox

Catching moonbeams

J. B. Pendry

PHYSICISTS at the City University, New York, have shown how it is possible to trap individual photons of electromagnetic radiation. A. Z. Genack and N. Garcia (*Phys. Rev. Lett.* **66**, 2064–2067; 1991) achieved this by passing microwaves into a disordered jumble of metallic and dielectric spheres. The details of their results show that the trapping, also seen with electrons in disordered semiconductors, occurs by a mechanism that has yet to be elucidated.

Every physicist pays lip service to wave-particle duality by which we can observe either particle-like or wave-like properties of matter provided that we do not

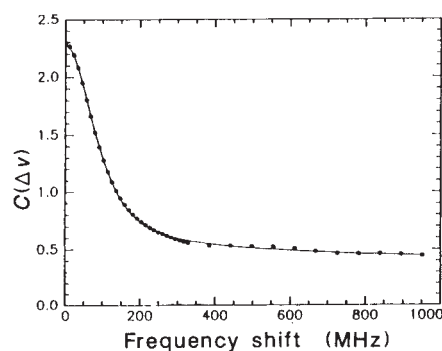


FIG. 1 The correlation function $C(\Delta\nu)$ of the microwave spectrum of Genack and Garcia's cylinder with a metallic filling fraction of 0.35. The data are given by filled circles; the solid line is a theoretical fit by the authors. The form of the curve $C(\Delta\nu)$ is roughly speaking a characteristic linewidth for the spectrum.

violate Heisenberg's uncertainty principle. This states that the position and momentum of matter cannot both be precisely defined. Insofar as momentum is associated with wave-like motion, Heisenberg's principle embodies the duality paradox. If we choose to define position, we envisage point particles occupying specific locations; if we define momentum, matter loses its point-like character and is delocalized in the form of a wave. According to the rules we can, in principle, force matter into either state.

In practice we have a clear notion of when we are dealing with waves or particles: X-ray crystallographers assure us that they can locate atomic coordinates in crystals with extraordinary accuracy. In contrast we never speak of the position of electrons in a solid, but always of their momentum, and for those at the 'Fermi' surface (the most energetic in a system) we can measure momentum with great precision. This is the uncertainty relation at work in condensed-matter physics.

The deciding factor in the position-momentum battle is the mass of the particle: atoms are heavy, electrons are light. The ultimate featherweight amongst particles is the photon which has no mass at all and was the first of the particles to be described as a wave,

by Huygens in the seventeenth century. How ridiculous to imagine that photons can be caught in a trap! Yet this is what Genack and Garcia have achieved.

In their experiment, random mixtures of metallic and dielectric spheres were contained in a copper tube 7.3 cm in diameter, at a total filling fraction of 0.6 but with varying metallic filling fractions. The transmission characteristics of this material show effects due to trapping of the photons at localized sites within the sample. Microwave radiation was launched from a horn placed 20 cm in front of the sample, and two Schottky-diode detectors were placed 2.5 cm apart on the output surface of the sample. The transmission coefficient fluctuated strongly as the configuration of the spheres was altered, and great care was taken to obtain a true average over many configurations of the sample. This was achieved by tumbling the specimen about its axis and taking as many as 30,000 readings for each sample.

It has been known for some time, from the work of Landauer, Mott, Anderson and others, that electrons can be forced to abandon their predilection for momentum. Fill a semiconductor or an oxide with impurities to make a thoroughly disordered environment, and the chances are that electrons injected into such a material will stay put, bound to some impurity. This localization phenomenon has two striking consequences: one is that diffusion is massively inhibited because electrons cannot break away from their local sites. The other is more subtle and has to do with the energy spectrum of the electrons.

In an ordered material the electrons occupy a smooth distribution of momentum states with a correspondingly smooth and continuous distribution of energies. Not so in the presence of strong disorder, where the electron energy levels are sharply defined, and the energy spectrum consists of a series of very sharp spikes, one per electron. We invoke another uncertainty relation to understand how this is so, that between time t and energy E ,

$$\langle \delta E^2 \rangle \langle \delta t^2 \rangle \geq h^2 (4\pi)^2$$

The left-hand side is the product of the mean uncertainties squared for time and energy, the right-hand side includes Planck's ubiquitous constant h . By implication very sharp, well-defined, energy levels ($\langle \delta E^2 \rangle \sim 0$) are extremely long lived ($\langle \delta t^2 \rangle \rightarrow \infty$). An energy spectrum with sharp spikes implies that electrons are trapped for long periods of time in localized states.

It is this spiky nature of the energy spectrum that Genack and Garcia have exploited to detect localization of photons in their experiment. They subjected the microwave spectra of their jumbled spheres to an autocorrelation analysis to find a characteristic

linewidth (Fig. 1) for the localized states at a particular filling fraction. As the correlation function $C(\Delta\nu)$ of Fig. 1 is a measure of how spiky a spectrum is, it also is a measure of how long the photons are trapped for and of how easily they can diffuse through the cylinder. Thus the authors could measure the effective diffusion constant at all metallic filling fractions (Fig. 2). When the photons are locked into localized states, we expect the diffusion coefficient to become zero.

Here Genack and Garcia faced a problem. Photons have a trick up their sleeves not available to electrons: if there is any mechanism that can dissipate energy, the photons can simply be absorbed and vanish into thin air. In contrast, electrons have mass and charge which have to be conserved and are unlikely just to disappear. As a result, in any real system, the photons will not last forever

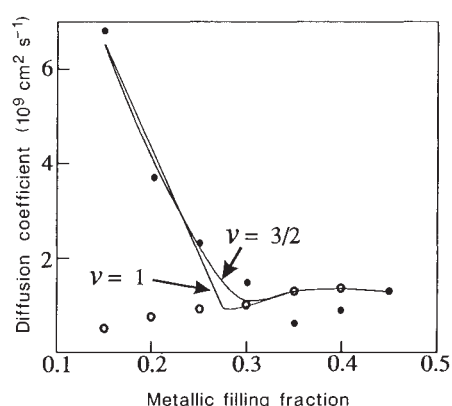


FIG. 2 The measured diffusion coefficient (●) and limiting value derived from the absorption coefficient (○) as a function of metallic filling fraction. The critical disorder arises at a filling fraction of 0.3. The solid lines are of the equation $D = \text{constant} \times (f_0 - f)^\nu$ with $\nu=1$ and $3/2$. Note that the latter gives the better fit, but that further data are needed near the transition, $f=0.3$.

but will eventually succumb to absorption. They will have a finite lifetime on their localized sites, giving rise to a residual effective diffusion coefficient. Hence the form of Fig. 2 — as disorder increases with metallic filling fraction the diffusion coefficient drops dramatically to a low, but non-zero value. Actually the authors have an independent way of estimating the absorption rate from their data, and the open circles show the irreducible diffusion coefficient this implies. Thus Fig. 2 shows a convincing demonstration that the non-absorptive component of diffusion drops to zero at a metallic filling fraction of around 0.3.

All this would be no more than an exercise in elegance were it not for the fact that it is far from settled what actually causes localization in disordered media. In the limit of weak disorder, particles move around in a diffusive manner which is well understood from existing theories. At stronger disorder, conventional perturbation theory breaks down and with it much of our understanding collapses. What is known is that there is a threshold of

HUMAN GENETICS

Breaking the fragile X

Kevin Davies

disorder beyond which the particles switch into localized states, and the diffusion coefficient vanishes at the critical disorder f_0 as,

$$D = \text{constant} \times (f_0 - f)^\nu$$

where f is some measure of the disorder, such as the metallic filling fraction in the present instance. There are many questions to answer in this critical region of disorder. What is the qualitative nature of the wave field? How strong are fluctuations in the transmission coefficient? What is the frequency dependence of transmission?

One key quantity is ν , the critical exponent with which the diffusion coefficient vanishes. Whether or not one can predict ν is the acid test of a theory. Some time ago P. W. Anderson and coworkers developed an imaginative theory, based on notions of scaling, which makes a clear prediction that $\nu=1$. Experiments on electron systems sometimes show $\nu=1$ but other specimens give $\nu=1/2$. A difficulty with these results is the charge an electron carries. Localization theory assumes that these particles do not interact and it is hard to assess to what extent this assumption is valid in the experiments.

Theoretical waters were further muddled by numerical simulations (A. MacKinnon and B. Kramer *Phys. Rev. Lett.* **47**, 1546–1549; 1981), for an ideal system of particles defined to be noninteracting, which obtained the exponent, $\nu=3/2$, a totally unexpected and as yet unexplained result. In using microwaves, Genack and Garcia have an excellent system for testing theories: although there are problems arising from the absorption, well controlled by the authors, the particles are noninteracting, as they are in the calculations. Alongside the data in Fig. 2 are shown two fits of the data — one with an exponent ν of 1 and one with an exponent of $3/2$. The latter has the closer fit, with a mean standard deviation half that of the former. It is fair to say that we really need a few more of these remarkable experiments before the argument is settled. And even if the exponent of $3/2$ is confirmed, why it arises is still a complete puzzle.

When a physicist seeks to understand natural phenomena, whether they be the thermodynamics of perfect gases, or in this case the interaction of waves with disordered media, progress generally treads a well-worn path: establishing the experimental facts, developing a qualitative understanding (the 'physics' of the problem) and finally codifying in terms of equations which describe all aspects. The absence of experiments which can get to the heart of the problem in a well characterized manner has hindered theoretical development. The results of Genack and Garcia should hasten us towards understanding the physics of waves in disordered media. □

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In what is one of the more keenly contested areas of human genetics research, three groups from France, Australia and the United States, reporting separately in *Science*^{1,2} and, last week, in *Cell*³, describe progress in the characterization of the molecular abnormalities associated with the fragile X syndrome. In addition, in their paper in *Cell*, Verkerk *et al.*³ have identified a gene which, by virtue of its physical location, expression and (partial) sequence, looks as if it is intimately involved in the clinical manifestation of the disease.

Fragile X syndrome is the most common inherited form of mental retardation, affect-

to this general rule, however⁷). Isolating yeast artificial chromosomes (YACs) which spanned the fragile site, Mandel's group then resolved the methylation differences to a single CpG island (often found at the 5' end of genes) containing a cluster of rare-cutter sites⁸, including *Bss*HII and *Eag*I (see figure).

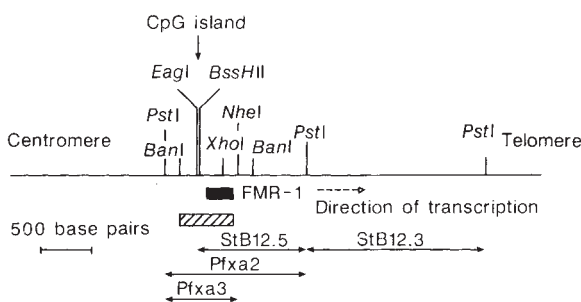
Oberle *et al.*¹ subcloned from the YAC encompassing the CpG island, and isolated a seven-kilobase *Eag*I–*Bgl*II fragment, StB12, lying immediately distal to the CpG island. A number of CG-containing restriction sites surrounding the CpG island were unmethylated in normal males and normal

transmitting males, but predominantly methylated in affected males. The region may be as small as one to two kilobases in length¹.

Even more striking, all three groups found a DNA insertion in the normal transmitting males, which is passed on to their daughters. Using StB12.3, Oberle *et al.* detected abnormally large *Eco*RI bands in these males, with increases ranging from 150–400 base pairs above the normal 5.1-kilobase restriction fragment. These insertions

were localized to a 550-base pair *Ban*I fragment¹ and, using *Pfxa*3, to an overlapping 520-base pair *Pst*II–*Nhe*I fragment by Yu and colleagues² (see figure). Daughters of normal transmitting males showed the same (or a slightly larger) insertion, but among individuals in the next generation who inherited the mutation, the insertion increased to 1.5–3.5 kilobases^{1,3}. In some individuals, several bands or smears were seen, implying somatic heterogeneity^{1,2}. Whatever the molecular nature of the mutation, whether it be amplification or insertion, it is usually, but not always, associated with the phenotypic expression of the disease. One immediate consequence of these findings will be to reinforce the diagnostic advantages of DNA screening for fragile X over current cytogenetic methods^{1,2}.

The presence of a CpG island near the fragile site suggests the existence of a nearby gene. Using the most distal cosmid from an overlapping four-cosmid contig spanning the fragile X region, Verkerk and coworkers³ identified overlapping positive clones, from a fetal brain complementary DNA library. The full length of the message is roughly 4.8 kilobases, of which 3.8 kilobases of cDNA has been isolated and sequenced. The gene, designated FMR-1 (fragile X mental retardation-1), has many interesting features in addition to its expression in the human brain (and placenta)³. It is highly conserved in most species, including *Caenorhabditis*



The region that has undergone mutation in the fragile X syndrome. Approximate positions of the restriction sites are indicated. Arrows refer to the subclones employed^{1,2}. Black box, FMR-1 exon³; hatched box, site of length variation^{1,2}.

ing an estimated 1 in 1,250 males and 1 in 2,000 females. Characteristic features sometimes also include large ears and testes, prominent jaw and altered speech patterns. The fragile site occurs near the end of the long arm of the X chromosome (Xq27.3), and can be induced under certain conditions in cultured cell lines. Because of variable penetrance, the pattern of expression in fragile X syndrome is extremely unusual: at least 20 per cent of males are clinically unaffected (normal transmitting males) but they can pass the trait on to their daughters, who are also asymptomatic. But members of the next generation are often mentally impaired, indicating that the fragile X mutation must pass through the female germ line before it can be expressed clinically. Perhaps the most attractive hypothesis to explain this pattern was put forward by C. Laird, who proposed that the initial DNA mutation, unseen at the cytogenetic level, blocks the reactivation of the inactivated X chromosome at Xq27 before oogenesis^{4,5}. In affected individuals, this 'imprinted' region would then be more methylated and less transcriptionally active than the normal chromosomal region.

Earlier this year, groups led by J.-L. Mandel and K. E. Davies found that rare-cutter restriction sites (rich in CpG dinucleotide sequences) were resistant to digestion in fragile X males, but not in normal males or normal transmitting males, due to hypermethylation^{6,7} (there were some exceptions

*elegans*³, under high stringency wash conditions. This means that alternative experimental strategies to study FMR-1 may be feasible, and could even complement the investigation of a family of nematode late-onset neuronal degeneration genes in elucidating the basis of similar human disorders⁹.

But of more immediate importance is the partial FMR-1 cDNA sequence³. Although the 5' end has not been isolated, the cDNA predicts an open reading frame of 657 amino acids, culminating with multiple stop codons, a long 3' untranslated tail, and a polyadenylation signal sequence. Close to the 5' end are 28 CGG codons interspersed with two AGG triplets, encoding a poly-arginine stretch. Comparable but shorter arginine-rich sequences are seen in histones and protamines. The basic nature of the predicted FMR-1 protein, and the presence of a consensus nuclear translocation signal, mean that FMR-1 protein could be a DNA-binding protein.

The 5' region of the FMR-1 cDNA, including the 30 CGG repeats, is encoded by an exon³ which maps immediately distal to the CpG island previously shown to exhibit abnormal methylation⁸, and within the one-kilobase *Pst*I genomic DNA fragment which detects the length variations in fragile X chromosomes^{1,2}. So the CGG repeat (also observed by the other groups^{1,2}) is a prime candidate for a role in the generation of the length variation at the fragile site.

As noted³, CGG repeats are found in the first exon of the X-linked androgen receptor gene¹⁰, where they encode a polyglycine sequence. Closer to the N terminus of the androgen receptor is a polymorphic run of about 20 glutamine residues in normal individuals, encoded by CAG repeats. But as La Spada *et al.* will shortly report in *Nature*¹¹, in patients with spinal bulbar muscular atrophy, an adult-onset motor neuron disease, the number of CAG repeats roughly doubles. This variation, which shows no size overlap between patients and controls, is tightly linked with the disease phenotype and is thought to be the causative mutation¹¹. Instability in similar regions may prove to be a common source of deleterious mutations.

In the space of just four months, researchers have moved from long-range pulse-field gels to a detailed map of the fragile site itself, and the discovery of what may be a bizarre two-step mutation mechanism. For a disorder that has long puzzled geneticists and clinicians, the pieces are starting to fit. □

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Comets in collision

David A. Weintraub

THE unexpected discovery of a circumstellar disk, possibly a protoplanetary disk, around the young, Sun-like star β Pictoris was one of the most exciting astronomical results of the past decade. Astronomers who now study the disk around β Pic have the opportunity to engage in one of the great pursuits of astronomy: to learn more about how the Earth and planets formed and to determine the likelihood that other planetary systems can form, have formed and are forming elsewhere in the Milky Way. In this spirit, Telesco and Knacke¹ now show that dust grains in the disk around β Pic may share an important similarity to cometary silicates. In complementary work, Bruhweiler *et al.*² show that gravitationally bound circumstellar gas around β Pic flows both into and away from the star. Together, these two results lend new support to the theory that comets and cometary debris are responsible for the observed circumstellar dust and gas, and that the disk is the signature of the mopping-up process that occurs after a planetary system has formed.

Like the Sun, β Pic is a main-sequence star. It is twice as massive and ten times as bright as the Sun. The star's age is indeterminate — somewhere between a few million and one billion (10^9) years. The disk was discovered after observations³ made by the Infrared Astronomical Satellite (IRAS) revealed that this star is brighter than expected for a main-sequence star. The satellite's observations, in the 12–100 μ m range of the electromagnetic spectrum, showed that several common, main-sequence stars, including Vega and Fomalhaut, are surrounded by small dust grains (much less than 100 μ m across). This discovery came as a rather shocking surprise to astronomers who had believed that the circumstellar environments of stars are very clean: radiation pressure is very effective at pushing small dust grains beyond the gravitational grasp of a star; and a second process, the Poynting–Robertson effect, quickly causes small dust grains to spiral into the star. Together, these two processes should sweep a planetary system clean of dust in only a few million years.

Although the disk of β Pic should have been swept clean by now, leaving only planets, planetesimals, asteroids and comets behind, it includes a dusty disk extending out more than 1,000 astronomical units (AU) from the central star⁴ (that is, 1,000 times as far as the Earth is from the Sun). The population of small dust grains must therefore be continually replenished, perhaps from collisions of planetesimals, comets and asteroids and/or from sublimation of small grains off surfaces of large bodies.

Since the 1970s, β Pic has been known also to have a shell or disk of gas, within 1 AU

of the star. Spectroscopic studies revealed repeated episodes of plasma falling in towards the star⁵. Like the dust, the infalling plasma must be replenished since it cannot exist for millions of years. One intriguing explanation for this occurrence is that large planets in the β Pic system are still clearing the planetary zone of stray comets left over from the planet formation process⁶. The Earth's atmosphere and oceans, in fact, may have been created by cometary impacts that occurred during the late-stage clearing process in our own Solar System. Some of these comets become star-grazers and vaporize when they pass too close to the star. If all the infalling mass in the β Pic system is due to star-grazing comets, 10–100 such comets are needed per year⁷.

The story of β Pic, therefore, is one in which a protoplanetary disk has evolved at least part of the way to forming planets. The dusty disk is continually renewed from the debris of comet and/or asteroid collisions or from cometary activity, including outbursts similar to that seen from Halley (see the recent news report⁸). Meanwhile, some comets whose trajectories are perturbed by close encounters with planets or protoplanets become star-grazers and thereby become fodder to be consumed by the star, as part of the infalling plasma cloud.

Telesco and Knacke have detected emission from small (less than 10 μ m) silicate particles within 50 AU of β Pic. The silicate infrared emission feature is common in dusty astrophysical environments. It is usually a broad feature with a single peak near a wavelength of 9.7 μ m. This feature is uniquely different for comet Halley, however, which shows a second peak⁹ near 11.3 μ m. More detailed studies of the silicate emission feature toward β Pic may also show this double-peaked profile. High spectral resolution studies of this strong silicate feature and of other emission and absorption features in the 3–20 μ m spectrum of β Pic now appear feasible. Such future observations will serve as a tool for assessing the sizes, ages and thermal histories of the dust grains and may answer many questions regarding the origin of the dust grains, the possible presence of cometary bodies in

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HUBBLE SPACE TELESCOPE

Real science at last

David Lindley

ASTRONOMERS working with the Hubble Space Telescope (HST) are no longer pre-occupied with proving to the public that the flaws in the satellite's optics were not fatal. After six months of spectacular images derived from the telescope's testing runs, the first new scientific data are being gathered, and several unexpected observations were presented at a seminar* last month.

Much of the interest is in data obtained

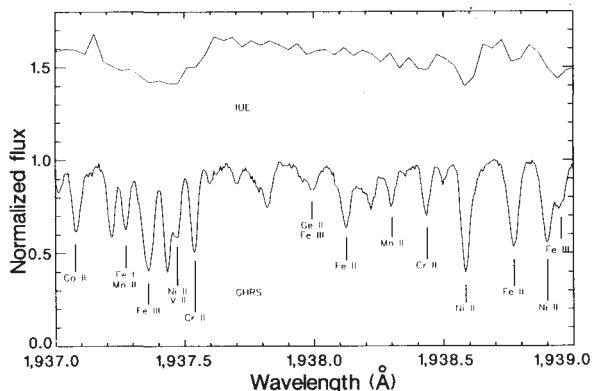


FIG 1. New (bottom) and old (top) spectra of χ Lupi.

by the HST's two spectrometers, the high-resolution and faint-objects spectrographs (HRS and FOS), both designed to operate primarily at ultraviolet wavelengths to which the Earth's atmosphere is opaque. The spectrum shown above (Fig. 1; D. Leckrone *et al.*, Goddard Space Flight Center), a 2-ångström-wavelength interval of measurements of the hot, chemically peculiar star χ Lupi, shows what the HRS can achieve in comparison the best that had previously been obtained by the long-serving International Ultraviolet Explorer (IUE). The greatly magnified spectral resolution turns a wiggly line into a forest of atomic identifications. The advantage of ultraviolet stellar spectroscopy is that it includes low ionization lines of almost all the elements, and when the elements in question are of relatively low abundance, only the low ionization lines may be

tion that gas flows both inwards and outwards at β Pic. Although both the rates of mass infall and outflow are variable, the largest measured infall event is 50 times more massive than the largest of the two observed outflow events. Although these observations demonstrate that some of the circumstellar plasma may be of stellar origin, they also show that stellar pulsations cannot account for all of the gas. Star-grazing comets are still the only viable explanation for producing much of the infalling gas. $\square$

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detectable at all.

Further spectra of γ Lupi turned up some novelties, and deepened some previous mysteries. Traces of ruthenium and arsenic were found, the first discovery of these elements in a non-solar astronomical spectrum. And the abundances of mercury and platinum, already known to be anomalously large, were estimated at 10^5 and 10^4 times solar abundance, respectively. The puzzle is that γ

Lupi has the brightness and colour of a relatively young, hot massive star, but has the heavy-element abundances that would be expected of a cool, highly evolved star after a lifetime of nucleosynthesis.

Spectroscopy also produced results of important but uncertain cosmological significance. For some years, observations from the ground have shown up dense patterns of absorption lines in the spectra of quasars, the most distant of astronomical objects; the lines have been

attributed to the presence of hydrogen clouds at intermediate distances. The further away the clouds are, the more their Lyman- α absorption line is redshifted from the ultraviolet towards longer wavelengths in the optical waveband, by cosmological expansion. Optical observations have suggested that the density of Lyman- α clouds decreases to shorter wavelengths, meaning that closer and therefore older clouds are less common than more distant and therefore younger ones. This hinted that the clouds were some sort of leftover from galaxy formation.

But measurements from both HRS and FOS found over a dozen Lyman- α clouds in a section of the ultraviolet spectrum of the bright quasar 3C273 in which only one would have been predicted by extrapolation from previous results (Ray Weymann, Mount Wilson and Las Campanas Observa-

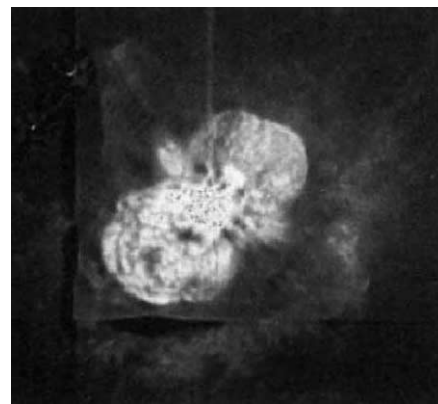


FIG 2. η Carinae, as seen by the HST.

tories). Before HST, no instrument had had both the sensitivity and spectral resolution to search for Lyman- α clouds in the ultraviolet; a tentative identification of one absorption line by IUE turned into several lines in the HRS data.

The quantity of hydrogen implicated in a typical Lyman- α cloud is a column density of about 10^{14} atoms per cm^2 — a nanogram, spread out over an unknown but presumably large diameter. Even clouds close by, identified by HST spectroscopy, will be impossible to detect in any other way, so that their cosmological origin and significance must be deduced from the most meagre of data.

These spectroscopic results contained most of the latest news, but no HST seminar would be complete without images. The bright star η Carinae provides a most striking ‘before and after’ comparison. The star is of historical note: it flared from a nondescript 6th magnitude to a remarkable -1, only just less bright than Sirius, in the middle of the last century, then faded over the next decades back to 6. The best guess at the structure, building on what was previously known of its history and on barely resolved images from ground-based telescopes, was that the star shed a lot of mass violently, creating two bright lobes of hot gas pushing out along what was presumably the polar axis, along with a few other scraps of debris.

The new HST observations (Fig. 2), however, turn this picture not upside down, but through 90 degrees. The WFPC (Wide-Field/Planetary Camera) image shows the lobes but reveals seeming jet-like structure at right angles to them (J. Hester, California Institute of Technology). On one side, the jet seems to run into a shock, presumably when it collides with the interstellar medium, and behind it is a curious ladder of bright lines, possibly some sort of standing wave. On the other side, the jet seems to have encountered a higher density of material, and pushes out a sort of ‘mushroom cap’ of expanding material. Regardless of all these details, the interpretation now is that the jets signify the polar axis, and that the bright lobes represent an equatorial torus seen in cross-section. $\square$

David Lindley is Associate Editor of Nature.

Tell tails

ICARUS, legend alleges, burned out by coming too close to the Sun. So did Icarus the comet, according to D. K. Yeomans (*Astr. J.* **101**, 1929–1928; 1991). Icarus and Apollo are two of several minor planetary bodies, studied by Yeomans, previously identified as stray asteroids owing to their eccentric Earth-crossing orbits. But it has always been recognized that some of these wayward inhabitants of the inner Solar System may be extinct comets, exhausted of volatile ices after countless orbits. Yeomans compared the orbits of several of these bodies with those to be expected if they were moving solely under the influence of gravity. Most behave as expected, but Icarus and Apollo do not. It seems they contain residual traces of ices that evaporate in rocket-like jets as they approach the Sun.

The eyes have it

A PARAMECIUM studied by Y. Nakaoka *et al.* (*J. Cell Sci.* **99**, 67–72; 1991) knows light from shade and makes an avoiding response when the wick is turned up or down. In both cases a measurable membrane depolarization ensues. Deciliated paramecia by contrast respond only to an intensity increase, which implies that the cilia, as well as the cell body, must be light sensitive. The creatures were known to possess retinal, but now it has been found that an antibody against frog rhodopsin labels all the membranes, including those of the cilia. Even if the antigen has a higher molecular weight than animal opsin, the cilium might be seen as a sort of proto-rod or cone and a fit model for vertebrate vision.

Anything goes

THE recently identified heavy '17-keV' neutrino may be just what theoretical physicists have been waiting for. Seen as an annoying anomaly for the standard model of particle physics, the neutrino could solve an even older anomaly, the charge of the electron, or so suggest R. Foot and S. F. King (*Phys. Lett. B* **259**, 464–468; 1991). There are insufficient constraints in the standard model to show why the electron has the precise charge it does, or why neutrinos have none. Foot has previously shown that making certain assumptions about the exact character of the various types of neutrino solves that difficulty. Foot and King now show that this works if one of the neutrino types has just the mass (17 keV) seen in several experiments. The idea introduces fresh oddities — including a further neutrino several million times heavier. But without proper experimental constraints, anything goes in this story.

Rebirth of a star performer

M. J. Colston

A NEW twist in the long and chequered career of the human tuberculosis vaccine BCG is heralded by the reports by Stover and colleagues, and Aldovini and Young, that appear on pages 456¹ and 479² of this issue. Both sets of authors have used BCG (bacille Calmette-Guérin) as a vehicle to express antigen-encoding genes from other pathogens, including the human immunodeficiency virus (HIV), and demonstrate that such constructs are able to induce humoral and cell-mediated responses to the recombinant antigens. So one of the oldest vaccines still in use may well take on a fresh lease of life, the old stager re-emerging as a star performer.

Albert Calmette and Camille Guérin first produced BCG in the early 1920s, by growing a strain of the virulent bovine tubercle bacillus on a medium containing potatoes, bile and glycerine. After many passages it was found to have lost virulence, being unable to produce tuberculosis in animals, and in 1924 it was declared to be a *virus fixe*. Several decades of controversy ensued, with disputes about its effectiveness against tuberculosis and even scandal surrounding its safety (from which it was subsequently exonerated).

Widespread administration of BCG became accepted policy in many countries in the 1950s and 1960s, following the demonstration of its powerful protective effect against tuberculosis in British schoolchildren³. It has even found a part in cancer therapy, where its remarkable immunostimulating properties have been used for immunotherapy. The recent observations of its poor efficacy against tuberculosis and leprosy in southern India^{4,5}, and the questioned cost-effectiveness of continued vaccination against tuberculosis in some Western countries, had led some to argue that its days were numbered. But BCG is safe, cheap and undoubtedly has a significant impact on the incidence of tuberculosis in some populations, particularly when administered early in life, and it is likely to remain part of the vaccination armoury for the foreseeable future.

The stimulus for the fresh activity centring on BCG has been the application of molecular genetics to the mycobacteria. These are particularly difficult organisms to work with, being slow growing (experiments with *Mycobacterium tuberculosis* often take three to four weeks, compared to 24 hours for similar experiments with *Escherichia coli*), and until recently they have been avoided by bacterial geneticists. But their importance as pathogens in their own right and as models for other intracellular infectious agents has led to the development of methods for introducing DNA into mycobacteria, and the propagation and expres-

sion of such foreign genes^{6–8}.

Aldovini and Young² have now introduced genes encoding HIV proteins (Gag, Pol and Env) into BCG, using a promoter from a mycobacterial heat-shock protein (Hsp 70) to drive expression of the genes. Stover and colleagues¹ have also employed mycobacterial heat-shock promoters to drive expression of the foreign genes, and have developed two different systems for propagating foreign DNA in mycobacteria — a multicopy plasmid system for extrachromosomal replication of the foreign DNA, and a system which uses the attachment site and integrase gene of a mycobacteriophage to achieve site-specific integration into the mycobacterial chromosome. These systems have been used to express β -galactosidase, HIV proteins (Pol, Gag, reverse transcriptase, gp20 and gp41) and tetanus toxin in BCG.

Both groups report that, when mice are immunized with recombinant BCG, antibody and cell-mediated responses against the foreign antigens are readily detectable. Both helper and cytotoxic T-cell responses were induced, and preliminary results indicate that the recombinant antigens can be recognized by T cells in association with both class I and class II molecules of the major histocompatibility complex. Thus all the immunological components required for generating immunity against a wide range of pathogens could be detected.

The success of BCG as a vaccine has been thought to be due to its ability to survive for long periods of time in the vaccinated host, thereby providing constant antigenic stimulation; this notion is supported by the observation of Stover *et al.* that killed BCG expressing tetanus toxin failed to generate an immune response against the recombinant antigen, whereas viable recombinant BCG did so. Paradoxically, this phenomenon might represent a limitation of the approach, at least in the short term; the many millions of people throughout the world who have received conventional BCG may eliminate a second (recombinant) vaccination too quickly to permit adequate priming of the immune response to the recombinant antigen.

If BCG is to be successfully developed as a recombinant 'carrier' of genes of other pathogens, a number of other issues remain. First, it will be necessary to identify the essential protective components of the pathogens, so that the appropriate genes can be transferred; for many pathogens, although a great deal is known about their immunological recognition, our understanding of the way that recognition is translated into a protective response is meagre. Second, many organisms undergo rapid changes as a means of avoiding immunological recogni-

tion. This is particularly the case with viruses, such as the influenza virus, and necessitates the constant change of vaccine strains to tackle new, pathogenic variants. Such an approach is unlikely to prove possible using BCG, because multiple vaccination results in severe reactions which would be unacceptable in many individuals. Finally, the conformational structure of the protective antigen might be important in some pathogens, and is likely to be influenced by its molecular environment or by post-translational modifications, both of which could differ when the molecule is expressed in BCG.

But although many questions remain, particularly those involving the relationship between immunological recognition and protective immunity, the new results point to NMR

an exciting opportunity to provide this tried and tested performer with a new and infinitely variable repertoire. □

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Diffusion and diffraction

Robert M. Cotts

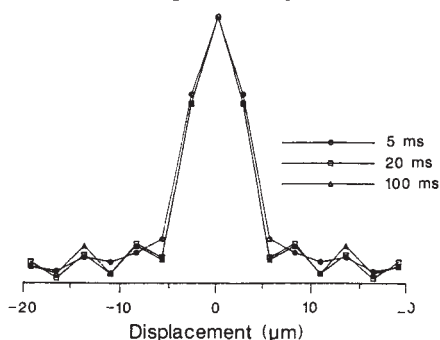
THE transport of fluids in porous media depends on the structure of cells or cavities and their interconnections, making this a central factor in heterogeneous catalysis, biological perfusion, percolation, permeation and so on. In studying transport, the interest is often in the spatial details of the typical unit structure of the medium rather than its exact location in the heterogeneous system. In an imaginative exploitation of the well-established NMR technique for measuring molecular diffusion coefficients, the pulsed-field-gradient spin-echo (PGSE) experiment, P. T. Callaghan *et al.* report on page 467 of this issue¹ NMR data that are analogous to the diffraction pattern of a disordered structure of pores and their interconnections for a model porous medium.

The analogy with diffraction is a mathematical one: Fourier transform techniques are used both in the analysis of the NMR experiment and in analysing X-ray, electron and neutron scattering studies of disordered structures. The new demonstration opens opportunities for applications of PGSE NMR that will yield structural parameters such as the mean pore spacing, its standard deviation, and the mean pore diameter as well as the intra- and inter-pore translational diffusion coefficients. The techniques will be especially useful in porous media having structural parameters between about 0.3 and 30 μm .

This new development follows more than a decade of progress since the early recognition² of the diffraction analogy in the context of NMR imaging; but these are not NMR imaging experiments. Cory and Garroway³ recently developed the formulation and applied the concepts in their demonstration determining the diffusion displacement profile of water in free (unbounded) diffusion and in restricted diffusion inside effectively impermeable yeast cells (see figure).

Independently, Callaghan *et al.*⁴ described the concepts and mathematical formulation of their proposed application of diffraction-like effects to PGSE NMR experiments on an interconnected porous medium.

All the new calculations and experiments built upon Kärger and Heink's⁵ definition of the diffusion displacement profile function



Diffusion displacement profiles of water in yeast cells at three different diffusion times. The characteristic cell width is approximately 5 μm . (From ref. 3.)

expressed as a Fourier transform of the spin echo's amplitude dependence upon field gradient pulse strength.

Use of magnetic field gradients to measure molecular translational diffusion coefficients in NMR spin-echo experiments dates back at least to the definitive 1954 paper of Carr and Purcell⁶ published less than a decade after the discovery of NMR. In the spin-echo experiment, a pulse of radio waves tips the nuclear magnetization vector 90° into the plane transverse to the applied magnetic field and its gradient. The gradient causes a distribution of precession frequencies that 'fans-out' or encodes the phases of spin vectors in the transverse plane. A second radio-frequency pulse 1–100-ms later tips every spin 180° and effectively reverses the distribution of precession frequencies, refocusing all spins into phase again a further

1–100-ms later. This simple, but elegant trick works because each nucleus is fixed in space so that its precession frequency, which depends on the local field, is constant. Translational diffusion destroys this order and causes a predictable attenuation⁶ of the echo signal amplitude, from which D , the diffusion constant, can be determined.

Roughly a decade later, Stejskal and Tanner⁷ showed it to be advantageous to pulse the magnetic field gradient in measuring molecular diffusion coefficients. Following their invention of PGSE NMR, the technique has been further developed and widely applied to homogeneous liquids, some solids and heterogeneous systems. The effects of bounded (restricted) diffusion, chemical exchange, sample-produced gradients, solvation, trapping, and so on, have been identified and studied in many circumstances. But it is only now that reciprocal-space (Fourier) reasoning has been applied to PGSE NMR.

In every PGSE experiment the diffusion time, Δ , is the time between a pair of field gradient pulses of amplitude g and duration δ . The first gradient pulse encodes the spins spatially as their local field is directly proportional to their coordinate along the applied magnetic field. The second pulse refocuses the spins after the time interval, Δ , for diffusive motion.

Without going into the mathematics, it is enough to note that the echo amplitude $E_\Delta(q)$ can be expressed in terms of an integral in which the integrand contains a phase factor, $\exp[i\gamma\delta g(z' - z)]$, where γ is the nuclear magnetogyric ratio, and $(z' - z)$ represents a possible spatial displacement during the diffusion time (i is, as usual, the square root of -1). Therefore the factor $\gamma\delta g$ is the equivalent of the wavevector q used in X-ray diffraction, roughly an inverse lattice spacing, and Callaghan *et al.*¹ use $q = (2\pi)^{-1}\gamma\delta g$.

Kärger and Heink showed that the Fourier transform of the echo amplitude, $E_\Delta(q)$, with respect to q , becomes the diffusion displacement profile⁴, $I[(z' - z), \Delta]$. This profile gives us the distribution of atoms along the field direction (relative to $(z' - z) = 0$) at the end of the diffusion time; it can be determined by varying the experimental field gradient while keeping the pulse delay Δ constant. Therefore structural information is contained in the q (reciprocal space) dependence of $E_\Delta(q)$. Cory and Garroway showed that, for pores or cells with impermeable walls, the echo amplitude $E_\Delta(q)$ can be expressed as being directly proportional to the power diffraction pattern of a single aperture whose transmission would be described by the shape function of the pore. Of course, there are no radio waves being diffracted in these NMR experiments. The analogy follows from the way the Fourier transforms in the NMR analysis resemble those used in wave-diffraction experiments. $E_\Delta(q)$ is, in this sense, equivalent to an average atomic form factor. ►

Callaghan *et al.* test their theory with a series of PGSE NMR measurements on the diffusion of water within an assembly of monodisperse solid polystyrene spheres in random loose packing. (They do not observe the NMR signal of the protons in the spheres.) Because they form a disordered lattice, the pores' spin-echo amplitude, as a function of q , resembles the X-ray diffraction pattern from a disordered solid. As they expected, the authors found a peak in the q dependence of $E_A(q)$ corresponding to $q \approx b^{-1}$, where b is the distance to the first nearest-neighbour shell of pores. Because b is a short distance, about 16 μm , high values of q are needed, so that a large field gradient was used, causing a large echo attenuation factor (about 100). Nevertheless, in what must be for this experiment a region of low signal/noise ratio we can see in the authors' Fig. 3 (page 468), the echo amplitude does go through a maximum at $q \approx b^{-1}$, rather than continuing to decrease, as the gradient strength is increased.

ENZYMOLGY

Lipases reach the surface

David Blow

NEW three-dimensional structures which help to explain how soluble enzymes can act at a lipid surface have emerged independently from three laboratories¹⁻³. The most recent of the papers, by Brzozowski *et al.*, appears on page 491 of this issue³.

In 1958, Desnuelle suggested⁴ that enzymes such as lipases must be considered as working in the two-dimensional surface of a micelle, rather than the three-dimensional volume of solution. In order to reach the surface the enzyme must be soluble, and Desnuelle proposed⁵ that a soluble enzyme might undergo a conformational change in fixing itself at the surface. These stimulating but speculative proposals are now given a degree of realism by structures for venom phospholipase A₂ (PLA₂) and for fungal lipase, complexed to hydrophobic substrate analogues¹⁻³, which demonstrate these structural changes.

The crystal structure data for soluble PLA₂ have exemplified the puzzle for long enough. Crystal structures of the free enzyme from the two main classes of PLA₂ (refs 6 and 7) exhibit close similarities, but neither gives any indication of a binding site for the long lipid chains of the phospholipids, so for many years the mode of action of these enzymes has seemed a mystery. A hint about the answer came in the publication last year of two independent crystal structures for bacterial and pancreatic lipases^{8,9}. Both of these structures showed a very obvious 'active site', containing the constellation of aspartic acid .. histidine .. serine familiar from the serine proteases. But this active site was buried inside the structure, and again gave no hint how a triglyceride substrate

In contrast to NMR imaging, which looks at the structure of one unit in a system, these PGSE NMR diffraction-like experiments, accept data from the entire porous medium and produce a diffusion displacement profile of the average unit pore and its degree of interconnection to surrounding pores. As the resolution can be as good as 1 μm , one can expect the technique to be applied to a wide range of heterogeneous systems. □

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might reach it.

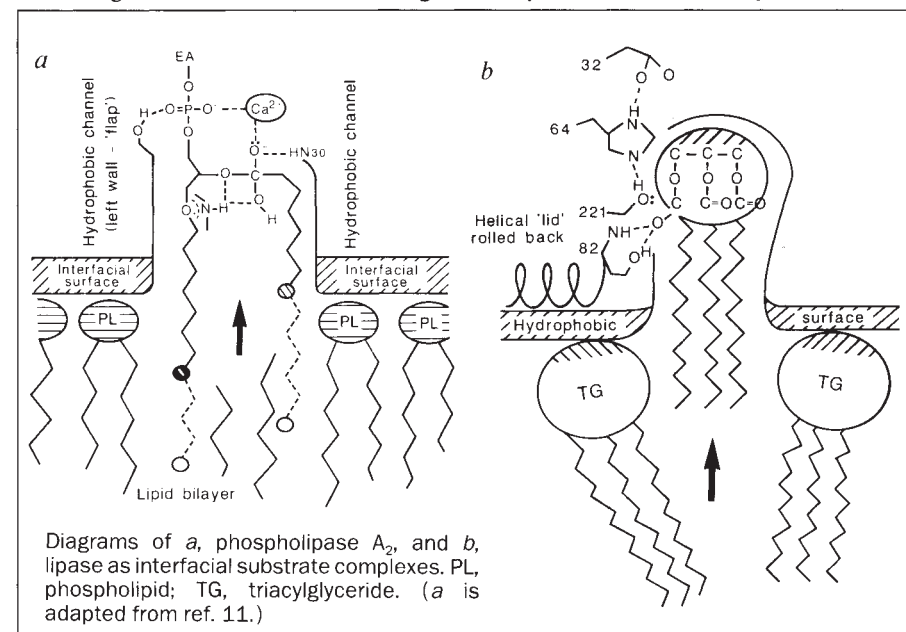
How, then, could a soluble enzyme carry a binding site for a lipid, and how could it attack a bond buried within a lipid membrane or a micellar surface? If the enzyme had such a lipid-bonding site on its surface, it would form a huge sticky patch, rendering it poorly soluble, and forced to aggregate, either with itself or with any hydrophobic surface it encountered.

The first real evidence for a structural rearrangement to reveal the binding site was found by Verger¹⁰, who measured a time delay for penetration of a micelle by PLA₂, which might include a structural change.

Crystallographic data now show how this change may happen. In free lipase and PLA₂ the site exists as a virtual site within the enzyme. In PLA₂, a hydrophobic channel is formed by the closure of a hydrophobic 'flap' composed of aromatic and aliphatic side-chains¹¹ (see figure). Substrate is bound without significant movement of the peptide backbone, as also shown by an earlier structure analysis of another PLA₂-inhibitor complex¹². In bacterial lipase a comparable hydrophobic binding site is opened up when a substrate is bound³, but in this case a major change occurs to the protein backbone, by movement of a seven-residue α -helix which lies over the active site residues as a 'lid', creating a new hydrophobic surface surrounding the active site.

Both of these binding sites are much smaller than the full size of the typical lipid substrate, and the crystal structure analyses used substrate analogues with hydrocarbon chains of only six to eight carbon atoms. Phospholipase A₂, a small molecule of relative molecular mass 13,000 (M_r 13K), is too small to bury a full-length membrane lipid chain of 15-20 Å. Sigler's group¹¹ suggests that the hydrophilic membrane surface provides a tight binding surface, or 'seal', to which the soluble protein attaches around the binding site. The diacyl-phosphatide substrate, carrying its polar head-group, is then drawn partly out of the membrane surface, making favourable polar interactions with a bound calcium ion and polar catalytic residues at the cleavage site, and allowing the proximal part of the lipid side-chains to interact with the 'flap', while the distal end still projects into the membrane. According to Scott *et al.*¹¹, the movement of the 'flap' (which probably occurs when the enzyme docks to the phospholipid surface) facilitates translocation of the substrate to the cleavage site.

Lipase attacks oily surfaces such as those of chylomicra and cannot rely on polar phos-



phorylated groups to form this seal. In the report in this issue, Dodson's group³ notes the creation of a non-polar surface encircling the active site, which may fulfil a similar role, making a hydrophobic 'seal' to allow the lipid to enter the active site without interacting with bulk water. This enzyme, too, will only partly withdraw the substrate, leaving its fatty side-chains projecting into the lipid.

A new crystal structure, published by myself and colleagues¹³, shows that bacterial cholesterol oxidase, another lipid-metabolizing enzyme, possesses an intact steroid-binding site which is contained within the surface of the soluble enzyme. The active site of cholesterol oxidase, an FAD-dependent enzyme of *M*_r 54K, is much further from the enzyme surface, implying that cholesterol must be fully withdrawn from the lipid surface into the enzyme. Preliminary results (Jiayao Li, P. Brick and D. M. Blow, unpublished observations) have identified a surface loop of the enzyme which is displaced when a steroid is bound. This conformational change once again suggests the possible formation of a surface which can seal to the phospholipid membrane.

These structures go a long way towards providing realistic models for Desnuelle's vision of a surface-active enzyme, changing GEOMAGNETISM

conformation from its fully soluble form as it binds to the surface. They create a framework for considering many questions which remain unanswered. What is the exact design of an enzyme surface which binds favourably to a phospholipid layer? How would the 'seal' form? What happens to the product of the enzyme reaction? If the substrate slides spontaneously out of the lipid into the active site, how does the product get away? How would the kinetics of such a system behave? Most importantly, the structures lead naturally to the design of experiments which might address these questions. □

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dipolar but assumed a more complicated geometry.

Since 1976, techniques have improved for separating multiple generations of magnetization from sediments such as those at Lake Tecopa. Indeed, a substantial reinvestigation of the Lake Tecopa section⁵ showed that the transitional VGP path, once properly isolated from the effects of secondary magnetization, lies about 90° west of the position shown by Hillhouse and Cox, and passes very close to the Californian sampling site. Although this revision did not modify the fundamental conclusion that the transitional field was not dipolar (the Lake Tecopa path is still distinct from the Japanese path), it was significant in another way.

Many of the palaeomagnetic records of the Matuyama-Brunhes transition available by the late-1970s had an intriguing site-dependent property; the VGPs for each record followed a path that passed near the sampling site. This pattern suggested that an axial multipolar (or 'zonal') field predominated during the transitional interval^{6,7} (Fig. 1). The original VGP path from Lake Tecopa had been a puzzling exception to the site-dependent pattern, but the new path agreed well.

The zonal hypothesis, being directly testable, stimulated a great deal of activity. New reversal records from volcanic rocks on land and sediments from the deep-sea revealed that the transitional field was often complex, with east-west (that is, nonzonal) components especially prominent during the latter stages of a reversal. The apparent complexity and variability led some workers to suspect that systematic features of transitional field behaviour, if they existed at all, might become recognizable only with more data. Doubtless, the uneven distribution of sampling sites, the variable filtering of the geomagnetic signal by sediments, and the very nonuniform sampling pattern offered by lava flows were all degrading the resolution of the palaeomagnetic record.

Given this background, one can appreciate the interest aroused by the latest findings of Tric *et al.*⁸, and Laj and colleagues in this issue². These workers have assembled a large subset of the data for the past 10 million years that shows a very distinct regularity; transitional VGP paths tend to lie near the broad longitudinal sector that includes

Reversals of opinion

Scott Bogue

THE remanent magnetism of rocks provides a rich history of the Earth's magnetic field, which has alternated in polarity throughout geological time. But what happens to the field during the brief 10³-10⁴-year intervals when it is in transition between its normal and reverse states? A current controversy centres on the one characteristic of reversals that should be most apparent in the palaeomagnetic record: whether the transitional field retains the dipolar geometry of its stable states. A landmark study in 1976 by Jack Hillhouse and Allan Cox¹ seemed to resolve this question, but improved analytical techniques and fresh palaeomagnetic data now appearing, including the Scientific Correspondence from Laj *et al.* on page 447 of this issue², have reopened the debate.

Hillhouse and Cox compared the remanent magnetism of two sedimentary sequences: one from the Boso Peninsula in Japan, the other from Lake Tecopa in southeastern California. The key strata were deposited and magnetized 730,000 years ago, during the Matuyama-Brunhes transition. This geomagnetic reversal, which took the field from reverse to normal polarity, is the youngest commonly found and is easily correlated from site to site.

If the magnetic field is dipolar at some instant during a reversal, then compasses (as recorded by the palaeomagnetic declination) at two or more sites will all point to a single

geographical position, a point termed the virtual geomagnetic pole (VGP). The ancient field inclinations, which provide a measure of the distance to the VGP from each site, will also correspond to this particular location of the VGP. Identical sequences of VGPs from distant sampling sites would strongly indicate that the field somehow rotated from one orientation to the other, remaining dipolar the entire time.

Two studies in the early 1970s concluded that the transitional field was dipolar^{3,4}, but Hillhouse and Cox drew attention to the newer results from California and Japan. The transitional VGPs for the two sites formed two bands separated by some 180 degrees of longitude. The clear implication was that the transitional field did not remain

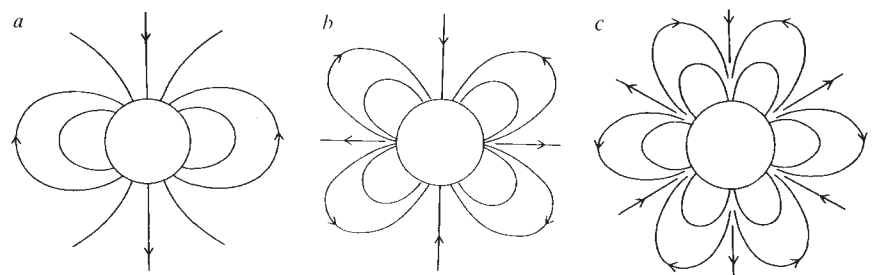


FIG. 1 Field lines for zonal magnetic fields: a, axial dipole; b, axial quadrupole; c, axial octupole.

North and South America (see the cover). The new transition path from Lake Tecopa, for example, passes near this region.

The clustering of the transitional VGPs not only revives the old hypothesis that the transitional field is predominantly dipolar, but also requires the sequence of intermediate dipole orientations to be similar from reversal to reversal. This persistence is intriguing. It hints that slowly varying inhomogeneities in the temperature or con-

ductivity of the lower mantle are affecting the much more rapidly varying dynamo process in the outer core. As Laj and colleagues point out, it may be significant that both primary-wave seismic velocity in the lower mantle and fluid flow in outer core are distinctive beneath the region traversed by so many transitional VGP paths.

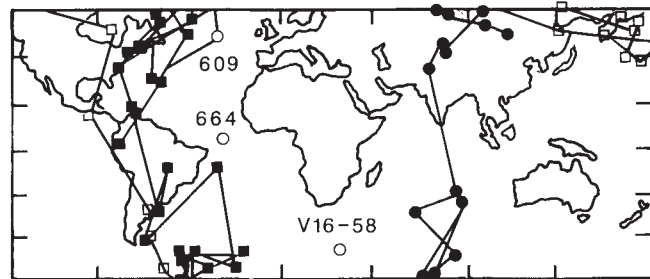


FIG. 2 Map after Clement and Kent⁹ showing antipodal VGP paths for the Matuyama-Brunhes reversal. Sampling sites are shown with open circles. The authors' conclusion: the transitional field was not dipolar and was not axially symmetric.

The VGP paths also show a preference for the longitudinal band on the opposite side of the globe, between India and Australia. This observation certainly complicates the simple dipolar interpretation, and the Matuyama-Brunhes reversal illustrates how. Clement and Kent⁹ compare reversal records from the floors of the Atlantic and southwest Indian oceans at latitudes 49° N, 0° and 46° S. The transitional palaeomagnetic directions from the mid-latitude sites have the familiar dipolar look: the VGPs for both track through the Americas. As viewed from the Equator, however, this same transitional field produces a VGP track across India (Fig. 2). The dissimilar paths are not contradictory, but rather are evidence that the transitional field was strongly nondipolar. Clement¹⁰ demonstrates that an equatorial octupole field, for example, can produce antipodal VGP paths like those observed.

A second example is provided by Tric *et al.*⁸. Their list includes seven records of the lower Jaramillo reversal, a reverse-to-normal transition that preceded the Matuyama-Brunhes by several hundred thousand years. The data all come from mid-latitude sites, three in the Southern Hemisphere and four from the Northern. Five of the sites show VGP paths centred over the Americas, but two, from either side of the Equator, show paths that lie about 90° further west. Again, a nondipolar transitional field is much in evidence. Further-

more, it is not like the transitional field of 200 thousand years later; VGP paths for the lower Jaramillo and Matuyama-Brunhes reversals from a single site in the North Atlantic are strikingly different¹¹. This short-term variability is not consistent with the idea that transitional fields are governed by lower-mantle structure.

Why is our new picture of the transitional geomagnetic field so different from previous ones? To some extent, the difference results from the many new data; the predominance of dipolar components during a reversal emerges only as a statistical tendency in this large collection. Could the pattern be fortuitous? Although 48 reversal records are included, only seven come from sites within 20 degrees of the Equator, and only five come from the Southern Hemisphere; geographical bias is still a worry. The scarcity of

sedimentary transition zones with accumulation rates greater than 5 cm per thousand years is another difficulty. There is reason to question the fidelity of the palaeomagnetic recording process in slowly deposited sediments, especially in light of the evidence that intervals of short-lived, rapidly varying field may occur during the transitional stage¹². It is even possible that persistent nondipole components, characteristic of the stable field, contaminate the remanence of such transition zones¹³.

Nevertheless, it seems unlikely that the regularity identified by Laj and colleagues is entirely spurious. So does deep-mantle structure find expression in the transitional field? Perhaps. But as Laj and colleagues readily admit, it is far from clear how inhomogeneities of the lower mantle affect flow in the outer core, or how a particular flow pattern translates into systematic features of the transitional geomagnetic field. □

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Good resolutions

PHOTOGRAPHIC film, says Daedalus, is a digital medium. A grain of silver halide in a bright region of the film has a high chance of being exposed and rendered developable; in a dark region, it has a low chance. After development, the resulting micro-mosaic of silver specks is a statistical digital sampling of the light-intensity distribution that hit the film.

This elegant chemistry has a singular flaw. An exposed grain becomes a speck of silver in the resulting negative, and you know where it is. An unexposed grain is dissolved by the fixer and you don't know where it was. In other words, the '1's of the digital binary code are recorded but the '0's are lost.

Daedalus now plans to remedy this defect. He recalls the fascinating physical chemistry of fossilization. As the organic remains of a dead organism slowly degrade and dissolve, their place is taken exactly by silica deposited from groundwater. So DREADCO chemists are inventing a photographic fixing solution supersaturated with silica. As it dissolves each unexposed grain of silver halide, it fills the resulting hole in the emulsion with a 'fossil' replica in white, light-stable silica. The negative thus records the '1's of the optical sampling as black silver grains, and the '0's as white silica grains. Even neater, the process can be inverted. The developed silver grains can be fossilized to white silica by a suitable solvent solution, and then the undeveloped silver halide grains are reduced to black silver. The result is a positive image with bright '1's and dark '0's, like the original scene. By elaborating the chemistry along established lines, with three layers of emulsion on the film each with its own colour filter, true positive three-colour photography should be possible.

'Fossilfilm'® will look distinctly sharper and crisper than existing products. But only elaborate post-processing will reveal its full power. Its grain structure of '1's and '0's provides for the first time a true binary statistical sampling of the original optical field. From a set of microdensitometer scans, computer analysis will reconstruct a smoothed, best-fit two-dimensional optical image for each colour layer. Even lens aberrations could be removed, by deconvolution via the known transfer-function of the imaging lens. The results will be an ultra sharp, grainless, undistorted picture, containing all the information on the original film. Detail comparable to the grain separation distance should be recoverable.

Such post-processing will be expensive, of course. Most of the time it will not be needed. But every so often, when a photograph turns out to be a unique record of some dramatic event, or to contain crucial scientific or forensic evidence, Fossilfilm will come into its own. David Jones

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Geomagnetic reversal paths

SIR — New palaeomagnetic records of the transitional fields during geomagnetic reversals are bringing about a radical revision of ideas on the morphology of these fields. Recently, we and others have reported many new reversal records, a remarkable preponderance of which show the position of the virtual geomagnetic pole (VGP) moving along the longitude of the Americas or its antipode^{1,2} as the reversal proceeds (*a* in the figure). The VGP is by convention the south magnetic pole of the dipole field that would give the observed declination and inclination at a site; hence, comparison of VGP paths from different sites yields information on the morphology of the field. The records include reversals ranging from 100,000 yr to at least 10 Myr in age; the persistence of the observed pattern may therefore reflect inherent characteristics of the geodynamo.

Here we point out that the same bands of longitude picked out by the transitional VGP paths prove to be important in other geophysical observations. For example, they have

particular significance in models of the present magnetic field at the core/mantle boundary, as they mark the boundaries of the dominant non-dipole feature at low latitudes, between 90° E and 90° W³. In the frozen flux approximation, this feature reflects the pattern of fluid motion in the outer core — specifically, a strong westward toroidal flow from 90° E to 90° W at low latitudes (part *b* of figure). The source of the flow appears to be partly influx of fluid from higher latitudes and partly upwelling from depth; there is a similar but weaker flow eastward from 90° E to 90° W. The longitude bands preferred by the VGP paths are thus those along which north–south flow is seen in the core models.

The preferred longitudes also represent regions of fast seismic-wave propagation (and therefore low temperature) in the lower mantle, possibly related to subducted lithospheric plates (part *c* of figure)⁴. The VGP paths therefore link the morphology of the transitional fields not only to features of the

present-day field and core flow patterns, but also to temperature anomalies in the mantle.

Although we do not yet understand the nature of these links, we can suggest some possibilities. As the poles of the dipole field are the foci of radial flux lines entering and emerging from the core, the VGP paths during reversals should tell us something about the behaviour of the flux bundles. The flux bundles are reduced in strength compared with times when the field is not reversing, but the VGP paths suggest that some vestige remains throughout the reversal. Given the core flow pattern, it seems likely that flux bundles will, at least initially, move towards the Equator at those longitudes where there is flow towards the Equator. Similarly, during the recovery phase, flux bundles should be transported poleward at longitudes where there is poleward flow. The longitudes of north–south flow are at 90° W and 90° E, which are indeed the preferred longitudes of the VGP paths.

The degree to which the field is dipolar during the reversal, or displays some more complicated geometry, with paths dependent on the observation site, will depend on the details of the radial flux bundles. It is not at all clear how intact the flux bundles will remain, nor how important diffusion will

be. For example, does some of the radial flux get swept into the toroidal flow? Do the flux bundles remain antipodal during the reversal? We think that it is not simply a coincidence that the longitudes of north–south core flow are those preferred by the VGP paths.

The link between reversal paths, the present magnetic field, flow in the core and temperature in the mantle, if confirmed by further work, will have profound implications. Because the time constants of mantle convection are orders of magnitude longer than those of the core, the persistence of the VGP bands over tens of millions of years suggests that fluid flow in the core is controlled, or at least modulated, by the temperature at the core/mantle boundary, which is determined by the pattern of mantle convection⁵. Thus, the engine of the planet appears to manifest itself in related internal motions from the core to the lithosphere, and geomagnetic phenomena in the core are ultimately related to plate tectonics.

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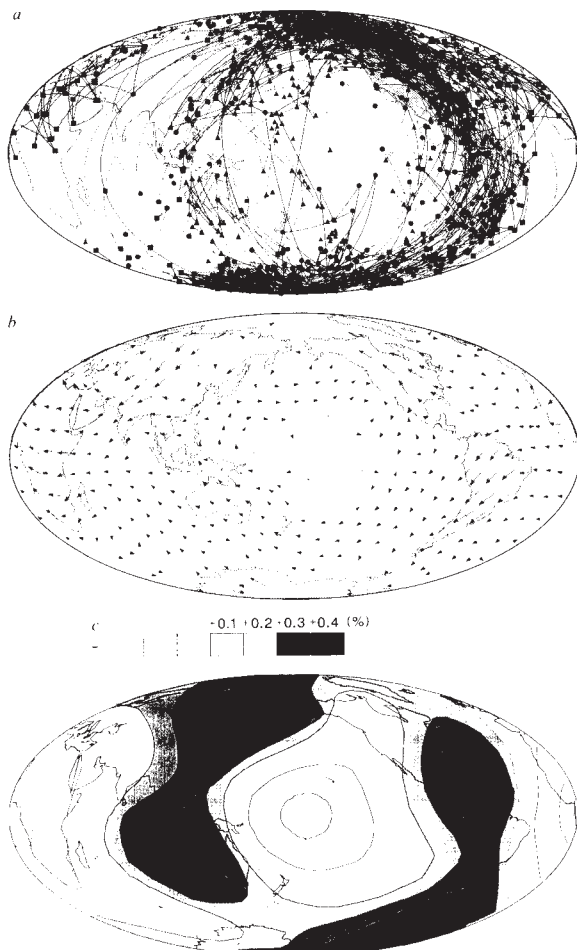
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Triple helix stabilization?

SIR — There is an error in the interpretation of the data in the recent paper by Reaban and Griffin¹. They demonstrate that when a plasmid containing a 2.3-kb segment of IgA switch tandem repeats is transcribed *in vitro* in the same direction as it is *in vivo*, the synthesized RNA forms a heteroduplex with the plasmid DNA, which results in a reduction of the supercoiling of the plasmid. This does not occur when the same sequences are transcribed in the antisense direction.

The authors interpret this to suggest that the switch sequence forms a triple-stranded DNA structure, which is stabilized by the RNA. But the RNA transcribed in this direction from the plasmid and also *in vivo* would be purine-rich, and not pyrimidine-rich, as they suggest, and therefore would probably



a, VGP trajectories for the Blake event (115,000–120,000 yr before present), the Upper Olduvai reversal, (~1.8 Myr) and two reversals at ~6.5 and ~11 Myr. *b*, Fluid motion at the top of the core (from ref. 3). *c*, Seismic P-wave velocity of the lower mantle, at a depth of 2,300 km (adapted from ref. 6). Velocities given as deviations from the mean. (See cover for colour version.)

not stabilize a triple-stranded DNA structure, as this plasmid has been shown to form a triple-stranded structure consisting of one purine strand and two pyrimidine strands, with the free single-strand being the purine strand².

This error does not diminish the value of the observation¹, as the finding of a heteroduplex and altered conformation of the DNA in the transcribed plasmid suggests a hypothesis for how transcripts from unrearranged heavy-chain constant-region genes could direct switch recombination.

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REABAN AND GRIFFIN REPLY — Further DNA sequence analysis of the plasmid pBS- α S2.3 confirms Stavnezer's observation that transcription by T7 RNA polymerase would produce a transcript containing the polypurine sequence (AGGAG)₂₈, rather than the polypyrimidine transcript reported in our manuscript¹. We regret this error in our data.

But the fact that the T7 transcript is purine-rich would not exclude it from stabilizing an intramolecular DNA triplex. Under our transcription conditions (8 mM MgCl₂ and pH 8.0) a purine-purine-pyrimidine intramolecular DNA triplex is likely to form. This triplex would be stabilized by base-pairing with the purine-rich T7 transcript via its excluded pyrimidine strand. A poly(dG)-poly(dC) sequence has been shown to form either a poly(dG)-poly(dG)-poly(dC) or a poly(dC)-poly(dG)-poly(dC) triple helix in a supercoiled plasmid, depending on the presence or absence of Mg²⁺, respectively³. The +Mg²⁺ triplex forms stably in neutral or slightly basic buffers, whereas formation of the -Mg²⁺ triplex is enhanced by low pH. The formation of dG-dG-dC (ref. 4), A-A-U (ref. 5) and 5'-TGGGGA-GGGGTGGGGAGGGGTGGGGAA-GG-3' purine-purine-pyrimidine (ref. 6) colinear triplexes under similar buffer conditions have also been reported. The base triplets predicted to occur with these triplexes have been observed in yeast transfer RNA⁷. Therefore our error does not alter our interpretation that the T7 transcript stabilizes an intramolecular DNA triplex.

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Whales and the military

SIR — Several live mass strandings of Goose-beaked whales (*Ziphius cavirostris*) have recently been reported¹ on the coasts of Fuerteventura, Canary Islands. In February 1985, 12 were stranded in the southern coast accompanied by a Gervais' beaked whale (*Mesoplodon europaeus*); in June 1986, four more were stranded alive on the northern coast, again with a single *M. europaeus*; in November 1988, three *Ziphius* and a single northern bottle-nosed whale (*Hyperoodon ampullatus*) were stranded on the southeast side (on the same date two pygmy sperm whales (*Kogia breviceps*) were stranded on the neighbouring island of Lanzarote). Military manoeuvres were observed at sea close to the stranding sites in February 1985 and November 1988 (ref. 1).

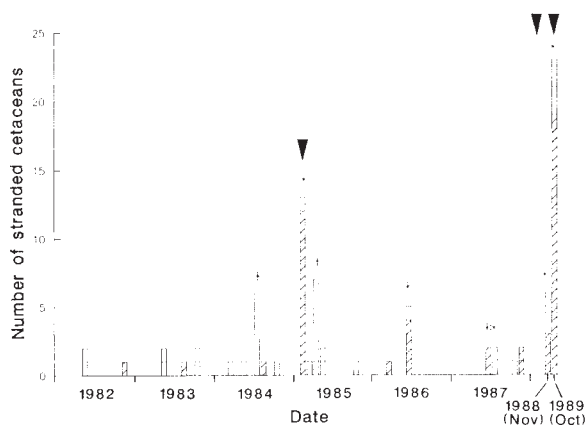
A further hitherto unreported mixed-species live mass stranding occurred on Fuerteventura in October 1989; three *M. europaeus* came ashore with two *M. densirostris* (de Blainville's dense-beaked whale) and many *Ziphius*. In total, about 24 animals are estimated to have stranded by M. Pizarro, R. Vonk and V. Martin. Again, the stranding occurred when naval vessels were clearly visible out to sea. Local people have only been aware of such military manoeuvres three times since 1985; on each occasion mass live strandings have occurred. No pathological examinations were conducted on any of the stranded animals but there were no apparent abnormalities or wounds. All the stranded animals soon died.

Strandings of *M. europaeus* on the east side of the Atlantic are very rare. Before the strandings in the Canaries only two others had been reported². There are also only a few reports of multiple deaths of *Ziphius* outside the Canaries¹; first, off Venezuela, when four decomposed bodies washed ashore on an island (the recorder considered that an underwater explosion, related to naval manoeuvres, might be responsible; second, when the bodies of three *Ziphius* and a striped dolphin (*Stenella coeruleoalba*) came ashore on the coast of Corsica with bul-

let holes in them; and third, a mass stranding, about which no further details are known, that occurred on the east coast of the United States. Two further small strandings are also known from the Galapagos and Puerto Rico.

Details of the general pattern of strandings in the Canaries from 1981 to 1987 have been noted by Vonk and Martin, and are presented with the mass strandings of 1988 and 1989 in the figure. The island of Fuerteventura is the closest of the Canary Islands to the African mainland. It is also the main island from which local people fish (off the east side) for squid. The sea between Africa and the island may, therefore, be an important feeding ground for toothed whales. (*Mesoplodon* stomachs have been found to contain hundreds of squid beaks.)

Reports of military interactions with cetaceans are rare, although sperm whales in the southeast Caribbean became atypically



Cetacean strandings in the Canaries, 1982-89. (The data were obtained by Vonk and Martin.) Shaded bars, *Ziphius*; open bars, other species; asterisks, live strandings; black arrowheads, military activity.

silent and then scattered when exposed to intense underwater, local, military sonar signalling, apparently from submarines³.

Naval manoeuvres off Fuerteventura may have stimulated an invasion of the island by ships coming towards the east coast through the whales' feeding grounds. This could have driven the whales shorewards and caused them to strand. Very little is known about the biology of *Ziphius*, so the reason for the unusual strandings can only be the subject of speculation.

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A scientific sampler

Mark Ridley

From Gaia to Selfish Genes: Selected Writings in the Life Sciences. Edited by Connie Barlow. MIT: 1991. Pp.273. \$17.50, £15.75.

CONNIE Barlow describes in her preface how her "childhood fascination for science" was "utterly quenched by schooling in science as facts", and she was only re-encharmed by the subject later, when she "chanced upon a lecture by James Lovelock". After that experience, she spent the next five years reading around in popular biology, particularly evolutionary biology, and had the idea of publishing together the more enjoyable passages she had come across.

From Gaia to Selfish Genes accordingly contains extracts from 33 authors. Each extract is quite short — the longest has 15 pages from *The Selfish Gene* by Richard Dawkins (Oxford University Press, 1976) — and gives the reader a flavour of each author, rather than following through the ideas in their complete form. The main authors include: James Lovelock who, together with some other more-or-less moonstruck writers, treats the Earth as "the largest manifestation of life"; Lynn Margulis, who writes about symbiosis; Arthur Koestler and Ludwig von Bertalanffy, on the errors (as they see them) of the mechanical conception of life; R. Axelrod and William Hamilton, on tit-for-tat; E. O. Wilson, on sociobiology; Richard Lewontin and others on biological determinism; and Richard Dawkins. There are shorter pieces by, among others, Leslie Orgel and Francis Crick, and by F. Doolittle and C. Sapienza (on selfish DNA); by Ashley Montagu (on sociobiology), and by John Maynard Smith (on 'the way forward').

Maynard Smith summarizes the topics in the book with the question, "How should biologists think about the relationship between wholes and parts — that is, between ecosystems and the species that compose them, between organisms and cells, or between cells and genes?" *From Gaia to Selfish Genes* might seem to hint at one appropriate direction for thought on the subject; but that is not Barlow's titular intention. As Maynard Smith says, "I do not agree with the theories proposed by Lovelock, Margulis and Gould, but I am fascinated by the phenomena they discuss."

Certainly, the extract from Lovelock here is unconvincing. He suggests that "the Earth is alive", and offers two arguments to support the proposition; one that the atmosphere is good (he says "optimal") for life, and another that living things provide feedback mechanisms which influence the atmospheric composition.



Richard Dawkins' coining of the word 'meme' in *The Selfish Gene* attracts the editorial eye of Connie Barlow in a chapter on the evolution of consciousness. The illustration is provided by "The heavy infantry of rebellious insects", Grandville's early nineteenth century engraving.

These atmospheric properties, however, are most likely to be consequential, as both Doolittle and Maynard Smith point out, and not adaptive. In Lovelock's own illustrative model, selection on individuals to control their body temperature has consequences for global albedo.

One general impression from the book is of misunderstanding, much of it unnecessary. We see Lewis Thomas shaking his wise head over unnamed "imaginative scientists" who maintain "that all sorts of behaviour,

animal and human, are governed exclusively by genes": and he is juxtaposed with an essay by E. O. Wilson which says that "human social evolution is obviously more cultural than genetic." And then, how could Lynn Margulis have persuaded herself that Dawkins' selfish genery is an "extreme" manifestation of the "reductionist view" that "biology is a subfield of chemistry and physics"? Where did Lewontin *et al.* find the "biological determinism" they criticize in the writings of the sociobiologists? Moreover, Lewontin *et al.*'s dialectical genetics, in which gene action depends on bodily context, looks to me exactly like the genetics of those politically less sophisticated biologists they believe to be "acting to preserve the interests of the dominant class, gender, and race."

Connie Barlow has edited the extracts carefully and the book is easy to read continuously from beginning to end. She intersperses comments to help the naive reader, and has packaged the prose with artwork illustrations, rather in the manner of the magazine *The Sciences* (Escher, Max Ernst, René Magritte, that sort of thing). The extracts are usually introduced by biographical material; the stuff about Lovelock and about Margulis almost amounts to a personality cult. It is mildly comic when these successful and much-honoured people try to pass themselves off as persecuted heretics, but the only truly ludicrous effect is provided by Carl Sagan, who appealed from a conference in Moscow to the clerics of the World "to join in an effort to save the planet". Whether they were persuaded is not recorded.

These personality cults fit in with Barlow's general aim, for she says she hopes "to encourage casual readers of nonfiction, devotees of popular physics, science majors, and students of the liberal arts to dig into the raft of appealing works in biology". My initial reaction is that any such readers who lay their hands on *From Gaia to*

Selfish Genes will already be familiar with the popular authors it includes; but I may be wrong. The US educational system is, I fear, all too successful in putting students off science in exactly the way Barlow describes, and if her anthology attracts any of them back it will have served a noble purpose. □

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Growing agenda

Daniel S. Greenberg

Ozone Diplomacy: New Directions in Safeguarding the Planet. By Richard Elliot Benedick. *Harvard University Press*: 1991. Pp.300. Hbk: \$33.50, £22.25; Pbk: \$13.25, £8.75.

SCIENCE and diplomacy became intimate in the postwar period, particularly in the quest for strategic arms control. The arms problem, though not resolved, is no longer as acute as it once was. But a relationship between the two professions is a durable necessity of our times. And the linkage has intensified with the recognition that environmental dangers transcend national borders and require multinational and often global efforts at control.

Richard Elliot Benedick, a career officer in the US Foreign Service, was at the centre of a historic event on this international front, negotiation of the 1987 worldwide agreement to limit and ultimately eliminate the production and emission of chlorofluorocarbons (CFCs) and other serious ozone-destroying chemicals. Benedick headed the US delegation in the lengthy, arduous and often hopeless-seeming negotiations that ultimately produced the Montreal Protocol on Substances that Deplete the Ozone Layer. Though the protocol's various loopholes and distant deadlines aroused various criticisms, it was nonetheless a landmark achievement of environmental foresight and commitment to action on a global scale. The serious threat posed by CFCs, reduction of the ozone shield against cancer-causing ultraviolet light, is not so immediate or direct as to set off popular alarms. Politics was deaf to scientific alarms in many countries. In environmental circles, Benedick is appreciated for helping keep his own government dedicated to the goal of CFC controls and for cultivating the international consensus that culminated in the agreement. In 1988, he received the Presidential Distinguished Service Award, the highest honour for career public service. But in the venerable tradition of the staff careerist, little is heard of author Benedick in his account of proceedings that stretched over several years of intricate dickering, rising and falling hopes and pragmatic compromises.

The negotiation of a pioneering international regulatory accord under the auspices of the anti-regulatory Reagan administration is surely a grand story of personalities, politics and scientific advice. But the Benedick version does not sing, let alone tell secrets. The author notes, for example, "While the US diplomatic efforts and the international negotiations were proceedings, anti-regulatory forces in the Reagan administration mounted a rearguard action in early 1987 to undermine the US position on protecting the ozone layer." Benedick states that

the last-minute resistance to ozone controls was justified on grounds that the US policy had not been cleared with several senior officials, including Reagan's science adviser, William Graham. Benedick states that "The reasons for this unexpected onslaught were uncertain." He speculates, however, that anti-regulatory forces had simply not been paying attention to the progress of negotiations. But one cannot help but suspect that the author knows more than he deems diplomatically appropriate for public discussion.

Although self-effacing, Benedick is generous to others in assigning credit for the successful outcome of the negotiations, especially to Mostafa Tolba, the executive director of the United Nations Environment Program (UNEP). Against a background of Third World suspicion of motives for pushing CFC curbs, and indifference among many industrialized nations, UNEP prodded and cajoled. Most important of all, the author states, the agency persisted in confronting the World community with the best available data, spotty as they sometimes were, and with forecasts of the fearsome risks of inaction.

Ozone Diplomacy is at its strongest in identifying the ingredients of the unprecedented agreement to eliminate CFCs. These are substances of great industrial, economic and social value, the chemical essence of the refrigerating and air conditioning that symbolize convenience and comfort in the rich nations and deprivation for the poor ones. Developing substitutes for these chemicals is proving to be a difficult task.

Benedick assigns the highest importance to generating scientific knowledge and conveying it to government officials. In working toward the ozone agreement, he states, "Scientists were drawn out of their laboratories and into the negotiating process, and they had to assume an unaccustomed and occasionally uncomfortable shared responsibility for the policy implications of their findings." In conjunction with scientific involvement, he continues, the political community had to support research and understand the findings and implications. The interplay between scientists and politicians was strongly influenced by the "power of knowledge and of public opinion," Benedick writes, pointing out that "A well-informed public was the prerequisite to mobilizing the will of governments and to weakening industry's resolve to defend the chemicals." Also of great importance, he found, was the presence of the multilateral UNEP, as both a forum for deliberations and prod to the participating nations. And he credits the role of national positions and leadership, noting that "The US government set the example by being the first to take regulatory action against the suspect chemicals." Nongovernment environmental organizations also played an important role in educating the public and promoting consensus, he states.

Though the 1987 Montreal timetables for CFC reductions were almost immediately

rendered obsolete by findings of an acceleration in ozone depletion, the diplomatic agreement embodied sufficient flexibility to provide for a speed-up in control production and use. But the latest findings, announced on 4 April, indicate that the ozone shield is diminishing at an even more alarming rate than was recorded just a year ago. Given the existing build-up of CFCs in the upper atmosphere, the cutbacks mandated by the Montreal agreement may come too late to prevent massive ecological damage.

Encumbered by listings of meetings and participants, *Ozone Diplomacy* is not a pleasurable text. But in mainly workmanlike prose, it records a critically important achievement in environmental cooperation. And it provides worthwhile insights on the harnessing of science and diplomacy, a partnership whose agenda is bound to grow. □

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Cross-channel traffic

N. Michael Green

Channels, Carriers and Pumps: An Introduction to Membrane Transport. By Wilfred D. Stein. *Academic*: 1990. Pp.326. £37.50, \$59.95.

THE introduction in the 1970s of single-channel conductance measurements on black lipid films and of patch clamping of membranes provided the ability to follow the opening and closing of single ion channels and to study the gating process. This led to advances in understanding which were well summarized in B. Hille's book on ionic channels, (*Ion Channels and Excitable Membranes*, Sinauer, 1984). The more recent major advance on the structural side has been the cloning of a whole range of membrane proteins which, in their roles as channels, carriers and pumps, control the intracellular environment.

Channels, Carriers and Pumps can be welcomed as a bold and on-the-whole successful attempt to integrate the structural and functional approaches to the understanding of membrane transport and intercellular communication. It gives a balanced survey of a wide field and should help to counteract the tendency for workers on each system to ignore their neighbour's progress. To achieve this, Wilfred Stein has made extensive use of results from cloning, sequencing, expressing and mutating ion channels and transport proteins to emphasize relationships between many of these systems at the molecular level. The cloning procedures are outlined and many of the sequences and their probable transmembrane distributions are shown. Although the structures are not yet

well enough defined to allow detailed interpretation of function, the results obtained by mutagenesis show clearly the directions in which progress is being made. Those not unduly inhibited by respect for the printed page should be able to keep up with the rapid advance for a year or two by annotation of the many diagrams provided.

Recent developments are well covered, often in separate boxed sections. This avoids disturbance to the general perspective, which is that of the author's earlier book, *Transport and Diffusion across Cell Membranes* (Academic, 1986), in which he emphasized the physico-chemical approach to understanding transport processes. Many illustrative calculations provide the reader with a feel for how transport systems operate in their cellular milieu. Basic concepts and experimental approaches are clearly explained, so that the general level is suitable for the advanced student, but for the research worker who wants to keep in touch with progress in related fields, there are many tables and diagrams to provide food for thought and an introduction to the appropriate literature. This concise and readable introduction should appeal to a wide audience. □

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Astronomy on the box

David W. Hughes

The Astronomers. By Donald Goldsmith. St Martin's Press: 1991. Pp.332. \$24.95.

POPULARIZING science is a Good Thing. The theory is as follows. The more members of the public understand what the scientists are up to, the more excited they become by the quest for discovery and the battle with the unknown. And so the more likely they are to put their hands into their pockets and fund the endeavour, or at least acquiesce to the government putting governmental hands into the pot that contains the communal taxes. To this end science on television is a Very Good Thing because it reaches a huge audience and its intricacies can be explained lavishly and excitingly.

Astronomy has been very lucky in this context. Not only is it replete with beautiful spectacles and ambitious space missions but it also has in its zoo some of the most exotic of creation's creatures. Neutron stars and black holes jostle with big bangs and gravity waves. The subject also has accomplished popularizers such as Patrick Moore and Carl Sagan. In 1980 we were treated, on both sides of the Atlantic, to Sagan's *Cosmos*, a television series that chronicled the historical develop-



The only astronomer most British TV viewers could name? Though Patrick Moore often points out that he's not a 'professional' scientist.

ment of astronomy. 1991 sees *The Astronomers* on the US Public Broadcasting Service television stations. The six hour-long episodes introduce the television audience to a supposedly eyewitness perspective of today's professional astronomers beaver away at the cutting edge of their science with state-of-the-art instrumentation and ideas to hand. A sum of \$5.3 million was awarded by the W. M. Keck Foundation to KCET-TV in Los Angeles, California, for the whole project. Donald Goldsmith, a professional astronomer and former consultant on the *Cosmos* team wrote the scripts. The book reviewed here is the book of the series.

Now it is rather difficult to divide modern astronomy into just six exciting topics, but I think Goldsmith has chosen well. His first topic concerns missing mass. Astronomers have slowly woken up to the fact that what they see is only a mere fraction of what exists in the Universe. Dark material abounds, and restrains the dissipation of galactic clusters and even makes our own Galaxy much more massive than we previously thought. Unfortunately, we have little idea as to what form this new material takes.

Next we have black holes, and here we rely on long base-line radio interferometry to produce maps of ever-higher resolution of the vicinities of these objects. The third major topic concerns the Big Bang and the detailed investigation of the spectrum and spatial variability of the remnant cosmic background radiation.

When looking for the next great astronomical breakthrough Goldsmith turns to gravitational radiation. If Albert

Einstein's general theory of relativity is correct, the movement of massive objects should result in the production of gravity waves. Ever more sensitive instruments are being built in the quest for their discovery.

Topic five brings us back to Earth with the investigation of stellar evolution. The book overviews modern theories of the birth of stars and the spectacular deaths of the more massive ones in explosions such as that of Supernova 1987A. Spice is added by an up-to-the-minute account of the search for the pulsar that is one of the last remnants of this astronomical catastrophe.

The last of the six sections stays mainly in our Solar System and relates the drama surrounding the Voyager 2 flyby of Neptune in 1989. Goldsmith also follows some of the more recent astronomical attempts to discover planetary systems around other stars.

The Astronomers is lavishly illustrated and what I liked especially was the way in which the procession of 'old favourites', of star fields, planetary surfaces and galactic vistas, was much enlivened by the inclusion of a large number of portraits of living astronomers. The text is rather too gushing. The thumbnail biographical descriptions overflow with complimentary adjectives. Surely all astronomers are not "eager, intelligent, helpful, hard-working, remarkable, renowned, compact, handsome . . ." Goldsmith seems to have moved in exalted circles. Many of the astronomers that I have met cover a much wider spectrum, and suggest adjectives ranging from diffident, uncertain, painstaking and reclusive to bombastic, overbearing, self-opinionated, rude and dismissive.

I also found the title of the book misleading. *The Astronomers* is really about US astronomers, and few foreigners grace the pages. I am not accusing Goldsmith of being xenophobic but it is quite clear that when he uses the word Cambridge he has in mind a leafy suburb of Boston, Massachusetts, and not an old university town in the English fens. Even when he visited the UK Infrared Telescope on Hawaii he picked one of the infrequent nights when it was being used by two Californians.

In essence, we are presented with an easily digestible popular romp through six of the highlights of the astronomy programme of the United States. □

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Moving story

Robert Simmons

Muscles and Molecules: Uncovering the Principles of Biological Motion. By Gerald Pollack. Ebner: 1990. Pp.300. \$55.

GERALD Pollack, author of *Muscles and Molecules*, believes in the conspiracy theory of muscle contraction. He quotes with evident approval another renegade, the late Graham Hoyle: "in other disciplines, proponents of different theories may fight bitterly, but they do attend the same meetings and coexist in unresolved strife until new facts reset the battle lines. In the small sub-discipline that is muscle, not only are the dissenters from current orthodoxy forbidden to appear, but also their work disappears from the record by never being quoted. Muscle scientists have a unique record for simply losing the facts."

Hoyle's own pet theory of contraction involved the generation of force by "super-thin filaments" which others found less often and less interesting than he did. Ironically, this theory does not even get a mention in Pollack's book. Indeed, Pollack is even more assiduous than the supposed conspirators at editing the record, and there is hardly a page that is representative of what most people in the field thinks.

Pollack believes that striated muscle shortens in a stepwise way, and he has often demonstrated this to his own satisfaction, but most agree that the observed effect is artefactual. Pollack now espouses the view of Bill Harrington that the driving force for muscle contraction arises from a transition from helix to random coil (melting) in the rod part of the myosin molecule. But this ignores the fact that myosin subfragment 1, which lacks the rod portion, supports motility.

Pollack's idea is that the thick filaments shorten in a stepwise way as the myosin rods melt sequentially along the filament, continuity being provided by connecting filaments which anchor the ends of the thick filaments to the Z-line. Melting is supposedly triggered by phosphorylation, in turn mediated by another component of the thick filament, C-protein — a candidate myosin light-chain kinase. But the evidence for a link between phosphorylation and control of contraction is flimsy, at least in vertebrate skeletal muscle.

Shortening of thick filaments is central to Pollack's theory. In the original formulation of the sliding-filament theory in 1954, there were the observations by A. F. Huxley and Rolf Niedergerke and by H. E. Huxley and Jean Hanson that the A-bands (made up of the thick filaments) remain of constant length during most active and passive length changes, and that the I-bands (made up of the thin filaments) shorten as the thin filaments slide between the thick filaments.

Pollack is fond of tabulating the number of papers supporting particular views, irrespective of their merit or relevance. He lists 28 papers published since 1954 showing more than 15 per cent shortening of the A-band and only 12 showing less than 15 per cent. But of 18 papers in the former category I managed to check, nine relate to *Limulus* muscle, generally agreed to be unusual. Of the remaining nine papers, there is not a single one that does not have a simple explanation for the observed A-band shortening, usually proffered by the original authors, and taken as a whole they are a thoroughly convincing body of evidence that the length of the A-band does not change under most physiological conditions.

The most flagrant example of misquoting by Pollack lies in the reproduction of micrographs by Alex and Françoise Fabiato, which indeed show A-band shortening, but without explaining fully that the (skinned) muscle fibre was allowed to shorten freely during maximum activation to reach the irreversible 'delta state', whereupon the A-band remained shortened after the fibre was released and then re-extended. The authors suggested that this might be an explanation of other reports of A-band shortening, citing one of Pollack's other references as an example.

Another of Pollack's contentions has been that the proportionality between isometric force and filament overlap, another of the major planks of the sliding-filament theory, is incorrect. In Pollack's hands, force does not fall much until overlap in nearly completely reduced. But when steps are taken to avoid potential artefacts arising from non-uniformities of sarcomere length along a fibre, a linear relationship is observed, and even he has had to concede recently that this is so. But he now states that this is true only for strictly isometric conditions and a small amount of shortening produces the alternative result.

In Pollack's theory, actin does not have a primary role and any interaction with myosin is incidental. Based mainly on his own electron micrographs he postulates that the myosin crossbridges form a lattice of A-bridges with neighbouring myosin molecules linked together rather than with actin, even, it seems, in rigor. Inexplicably, there is no reference to reconstructions of the insect fibrillar muscle lattice or decorated thin filaments, which clearly demonstrate the interaction between myosin and actin. According to Pollack, actin is permitted to slide by a ratchet-like interaction inside the cage formed by the A-bridges (or it may itself melt and shorten). The activation of the myosin ATPase by actin, one of the main pillars of conventional crossbridge theory, is dismissed airily, with the remark that other factors can activate the myosin ATPase. But none surely under physiological conditions and by the same basic kinetic pathway?

Again on the basis of his own electron micrographs, Pollack proposes that there are

I-bridges between thin filaments and connecting filaments. He finds fault with the conventional structure of the thin filament, with tropomyosin and troponin acting as integral control proteins, and instead places tropomyosin in the connecting filament, with troponin forming the I-bridges to the thin filament. The tropomyosin molecules melt during contraction too, apparently. Some 40 years of painstaking research on thin-filaments structure and function which supports the conventional view barely receives a mention.

Much of Pollack's theory of contraction will sink without trace if it can be demonstrated in motility assays that the force between individual nonphosphorylated myosin molecules and actin is similar to the force per myosin in muscle itself. Nor is Pollack's book likely to stimulate much research, as the real areas of controversy lie elsewhere. *Muscles and Molecules* represents a missed opportunity as the field would benefit from a full-length critical text and Pollack's views could have been incorporated into such a text in their proper context. The book is instead solely a vehicle for his own flights of fancy, and these prove to be insubstantial outside his own area of expertise and quirky within it. □

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New in paperback

Andrei Linde's *Particle Physics and Inflationary Cosmology* (for review see *Nature* 350, 666-667; 1991) is now available in paperback. The book is published by Harwood at \$29, £16. □

From Routledge comes *An Introduction to Visual Perception* by Nicholas J. Wade and Michael Swanson, which provides a useful discussion of the function of vision in a three-dimensional environment. Price is £9.99. □

For those with an interest in gastroenterology, C. E. Steven's *Comparative Physiology of the Vertebrate Digestive System* should make fascinating reading. The book is published by Cambridge University Press; price is £15. The factors generating species diversity and the genetic diversity of natural populations are the subject of *Genetic and Ecological Diversity: The Sport of Nature* by L. M. Cook. Publisher is Chapman and Hall; price is £13.95, \$29.95. □

Of use to all Earth scientists will be the *Earth Sciences Dictionary* from Chambers and edited by P. M. B. Walker. Price is £9.99. □ From Blackwell comes the paperback edition of *The Origins of Human Disease* by Thomas McKeown, which is a history of the diseases of humankind from the earliest times to the present day; price is £11.95, \$21.95. □

Christopher H. Scholz's *The Mechanics of Earthquakes and Faulting* is published by Cambridge University Press at £25. □

In his book *Dynamics of Dinosaurs and Other Extinct Giants*, R. McNeill Alexander uses simple physical principles to explain the life, locomotion and final extinction of these huge creatures. Publisher is Columbia University Press, price is \$15. □

The p53 tumour suppressor gene

Arnold J. Levine, Jamil Momand & Cathy A. Finlay

The cell cycle is composed of a series of steps which can be negatively or positively regulated by various factors. Chief among the negative regulators is the p53 protein. Alteration or inactivation of p53 by mutation, or by its interactions with oncogene products of DNA tumour viruses, can lead to cancer. These mutations seem to be the most common genetic change in human cancers.

MUTATIONS in the p53 gene are turning out to be the most common genetic alterations in human cancers. Yet until recently, little was known about even the normal functions of p53. Further, different studies have led to the confusion of its being classified variously as a tumour antigen, oncoprotein and tumour suppressor.

The nuclear phosphoprotein p53 was originally discovered in extracts of transformed cells, reacting with antiserum from animals with tumours induced by simian virus 40 (SV40)^{1,2}. The protein was found to form an oligomeric complex in SV40-transformed cells with the SV40 oncogene product, the large T antigen^{1,2}. Because large T antigen is needed to maintain the transformed phenotype, it was suggested that this interaction is important for transformation. Thus p53 came to be classified as a tumour antigen. Then large quantities of p53 (5–100-fold more than in nontransformed cells) were detected in a variety of tumour-derived or transformed cell lines in culture^{1–3}. In cells transformed by a temperature-sensitive SV40 large T mutant, both the amount of p53 and the appearance of the transformed phenotype were able to be regulated by temperature⁴. This regulation of p53 occurred after its translation, and in transformed cells the protein was found to have a much longer half-life than that in nontransformed cells^{5,6}. And a variety of genomic and complementary DNA clones of p53 were isolated that could immortalize cells in culture⁷ and cooperate with the *ras* oncogene to transform primary rat embryo fibroblasts in cell culture^{8,9}. Thus p53 came to be classified as an oncogene.

All of these transforming p53 cDNA clones turned out, however, to be mutant forms of p53 (ref. 10). Further, the p53 gene now looks like being a tumour suppressor gene, negatively regulating the cell cycle and requiring loss-of-function mutations for tumour formation: expression of cDNA or genomic clones of wild-type p53 suppresses the transformation of cells in culture by other oncogenes^{11,12}, the growth of transformed cells in culture^{13–17}, and the tumorigenic potential of cells in animals¹⁸; and deletions or mutations of wild-type p53 alleles occur in several animal¹⁹ and human²⁰ tumours.

The p53 protein has therefore changed from being classified as a tumour antigen to an oncogene product and now to a tumour suppressor. Wild-type p53, like the retinoblastoma gene product, seems to negatively regulate cell growth and division. But unlike Rb, some mutant forms of p53 gain a function so as to stimulate cell division. It is perhaps because of the gain-of-function mutations, which can act in the heterozygous state, that p53 mutations are so common in human cancers.

The p53 gene in human cancers

The p53 gene has been implicated in many inherited and sporadic forms of malignancies in humans. The human gene lies on chromosome 17p and has been examined in a wide variety of primary tumours, xenografts and cell lines derived from tumours. Of these, colon carcinomas have been studied in the most detail: 75–80% show a 'loss' of both p53 alleles, one through deletion, the other through a point mutation. The point mutations are usually missense, giving rise to an altered protein^{20–22}. A similar 'reduction to homozygosity' is seen at the

p53 locus in lung cancer^{23,24}, brain²⁵ and breast²⁶ tumours and chronic myelogenous leukaemia cells in blast crisis²⁷. This suggests that mutations (deletions) at the normal p53 locus are recessive to the wild-type p53 allele, contributing only to tumorigenesis when the wild-type allele is inactivated. (This could occur through errors of mitosis or recombination during mitosis that lead to the loss of the normal chromosome, as well as through mutations of the normal allele.) This is in keeping with the wild-type p53 gene being a tumour suppressor gene. Recessive mutations also underlie other types of tumours, such as retinoblastoma and Wilms' tumour.

Figure 1 shows a compilation of the position of point mutations in the human p53 gene from various tumours, xenografts and cells derived from tumours. The mutations have several features. First, most are missense mutations, giving rise to an altered protein for which there would seem to be positive selection. Second, the mutations selected for are not randomly scattered. The majority are clustered between amino-acid residues 130 and 290 (out of 393). And most of these mutations are localized in four regions of the protein (residues 117–142, 171–181, 234–258 and 270–286) which are highly conserved among several different species²⁸. Finally, there are at least three mutation 'hot spots', affecting residues 175, 248 and 273 (Table 1). The frequency and distribution of these hot spots differs among cancers from different tissue types, but it is not known why these differences exist. They could arise from the different mutagens or other aspects of the environment in the tissues, or from the different selective pressures for promoting cell growth. Nevertheless, it seems clear that different alleles can have different properties.

What of p53 mutations in inherited forms of cancer, such as Li-Fraumeni syndrome? This is a rare autosomal dominant syndrome characterized by diverse neoplasms at many different sites in the body. Affected families have a high incidence of cancer, and of the six examined so far, all have p53 mutations clustering between codons 245 and 258, with at least two inheriting mutations for codon 248 (ref. 29). These inherited mutations

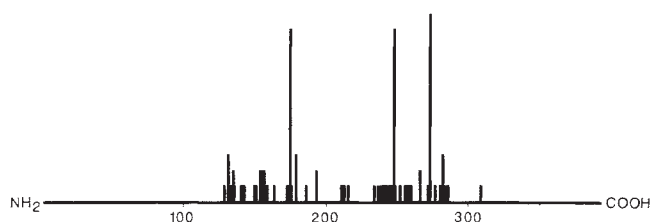


FIG. 1 The distribution of p53 mutations in the human p53 gene. The horizontal bar represents the 393 codons of the human p53 gene from N-terminal (NH₂) to C-terminal (COOH) ends. The vertical bars are the percentage of mutations that occur at each codon of the p53 gene as sequenced from 94 different primary tumours, xenografts or cell lines derived from tumours (brain, breast, colon, oesophageal, lung, neurofibrosarcomas, osteosarcomas, rhabdomyosarcomas, T-cell lymphomas). The highest percentage is at position 273 where there were 12 (13%) mutations. These data are compiled from 12 different sources in the literature^{20–27,29,53–61}.

are found in the heterozygous state in noncancerous cells of Li-Fraumeni family members, and do not interfere with the developmental process and normal functions of the individual bearing them. Rather, they could have a predisposing effect in carcinogenesis. Indeed, transgenic mice carrying a mutant murine p53 allele (along with two wild-type copies) have offspring with a much higher risk of developing cancer³⁰. If a mutant allele provides a growth advantage in the heterozygous state, then the number of cells with one mutation at this locus will increase, as will the probability of a reduction to homozygosity or of a second mutation arising in the cell harbouring the first mutation. This could explain why p53 mutations are seen so commonly in human cancers.

Although only six Li-Fraumeni families have been examined so far, the restricted distribution of their mutations at the p53 locus compared with those found in the wide range of sporadic cancers, lends further support to the idea that different p53 alleles have different properties.

Properties of mutant and wild-type p53

Several of the mutant p53 genes have been cloned and the resulting cDNA and genomic clones tested for their biological properties (Table 2). The wild-type allele can suppress the growth of transformed cells in culture¹¹⁻¹⁷ and the formation of tumours in animals¹⁸. The allele mutant for residue 175 is more efficient (3-10-fold) than the allele mutant for residue 273 in cooperating with the *ras* oncogene in transforming primary rat cells in culture^{31,32}. (Wild-type p53 has transformation-suppressing activity in a *ras* complementation assay.)

The mutant p53 proteins examined so far each have a much longer half-life than that of the wild-type protein³¹, resulting in large amounts of the mutant proteins in transformed cells³¹ and tumours²⁴. In these cells, the conformation of p53 with a mutation between amino acids 135 and 175 is different from that of the wild-type protein, as indicated by monoclonal antibodies^{33,34} and by the binding of the heat shock protein, hsc70³¹. Mutants for residue 273 have a normal or little-altered conformation. Similarly, both wild-type p53 and p53 with a mutation in residue 273 can transactivate a test gene^{35,36}. Murine p53 mutant for either residue 135 or 215 fails to transactivate in these tests. Table 2 reviews these observations; clearly, different mutant alleles of the p53 gene have different biological and biochemical properties. Perhaps cancer patients with different p53 mutant alleles have different prognoses.

Regulation of cell growth by p53

Wild-type p53 can suppress or inhibit the transformation of cells in culture by either viral or cellular oncogenes^{11,12}. Also, the introduction of the wild-type gene or cDNA into a transformed cell in culture stops cell growth¹³⁻¹⁵ at the G1 phase of the cell cycle, following mitosis. Wild-type p53 can even reduce or eliminate the tumorigenic potential of a cell line in culture¹⁸.

Through what mechanisms do mutant p53 proteins exert their effects? Different sets of experiments give us some clues. The first involves the p53 mutant for residue 135 (Ala to Val change in mouse) which is temperature-dependent, acting like the wild-type protein at 32°C and like a mutant at 37-39.5°C (refs 16, 17). At 32°C, rat embryo fibroblasts, transformed both by this temperature-sensitive p53 mutant and by *ras*, stop growing at late G1, failing to enter the S phase. The p53 protein in the mutant conformation at 37-39.5°C is localized to the cell cytoplasm^{17,37,38}, whereas at 32°C it acts as wild type, moving to the cell nucleus and stopping cell growth.

Also, cells transformed by both mutant p53 and *ras* have been obtained in which the amount of mutant p53 can be controlled by an inducible vector³⁹. Turning off the synthesis of mutant p53 in these cells results in a dramatic loss of their transformed phenotype, and they regain a normal morphology, with a reduction in their saturation density and plating efficiency (G. Zambetti, M. A. Labow and A.J.L., unpublished results). And a

mutant p53 allele under similar control and transfected into a primary rat embryo fibroblast, can negate the normal function of the endogenous wild-type p53 (ref. 17, and G. Zambetti, M. A. Labow and A.J.L., unpublished results).

Two hypotheses have been proposed to account for these results. First, the mutant protein could form an oligomeric complex with a wild-type subunit. With the mutant form in excess, this could prevent the wild-type subunit from functioning and therefore represent a dominant loss-of-function mutation. This idea accords with the requirement for a high concentration of mutant p53 to transform cells in culture¹⁰ and the formation by p53 of oligomeric complexes. In cells transformed both by the temperature-sensitive p53 mutant (Val 135) and by *ras*, the mutant forms a complex with the endogenous rat p53 (wild type) and keeps it in the cytoplasm, where p53 is unable to block cell growth¹⁷. A shift to 32°C results in the movement of p53 into the nucleus and a block in cell growth, indicating that some dominant loss-of-function mutations could work by regulating the entry into the nuclear compartment^{17,37,38}.

Alternatively, mutant p53 could gain a new function that overcomes the negative regulation by small quantities of wild-type p53. Support for this hypothesis comes from studies where a mutant p53 cDNA was introduced into a cell that did not express p53. Expression of the mutant p53 protein enhanced the ability of these cells to produce tumours in animals⁴⁰. Because there was no endogenous wild-type function for the mutant to eliminate, it must have been acting to stimulate cell growth (tumour formation).

Thus, there is evidence for both dominant loss-of-function mutations in transformed cells and gain-of-function mutations in tumorigenesis assays. The two are not mutually exclusive. Also, as overexpression of the wild-type protein in a cell with mutant p53 protein or other oncogene products suppresses transformation^{11,12}, cell growth¹³⁻¹⁷, and the tumorigenic potential of the cells¹⁸, the ratio of mutant to wild-type p53 in a cell could be critical in regulating cell division. And evidence that p53 mutations can confer a phenotype on and promote the growth of cells when heterozygous, rests solely on cell culture experiments¹¹⁻¹⁷, although it is hinted at by the high risk of cancer seen in the previously described transgenic mice and members of Li-Fraumeni families heterozygous for mutant p53.

Interactions of p53 with viral oncoproteins

Several types of DNA viruses can initiate tumours in animals. These include SV40, some adenoviruses and human papilloma virus (HPV) type 16 or 18. Each encode one or more oncogenes whose products promote the growth and division of the host cells. The oncoproteins (SV40 large T antigen; adenovirus E1A and the E1B 55K protein; HPV E6 and E7 protein) bind the retinoblastoma gene product Rb and p53, both negative regulators of cell growth (refs 1, 2, 41-45). That each virus encodes separate domains or proteins to bind Rb and p53 suggests that Rb and p53 do not act sequentially in a pathway where one is dependent on the function of the other. Rather, they could act in two distinct parallel pathways or redundantly (probably by both acting in late G1 to regulate entry into S phase). There was little proof that the viral oncogene products could inactivate p53 or Rb until it was demonstrated that the E6 protein (type

TABLE 1 Comparison of p53 mutations in cancers

Tissue	Number of hot-spot mutations at amino-acid residue:		
	175	248	273
Colon (<i>n</i> = 35)	8 (23%)	6 (17%)	6 (17%)
Lung (<i>n</i> = 29)	0 (0%)	1 (3%)	4 (14%)
Total tumours (<i>n</i> = 94) (refs 20-27, 29, 53-61)	11 (12%)	11 (12%)	12 (13%)

TABLE 2 Properties of p53 wild-type and mutant alleles

Property	Amino-acid residue (mutation)			
	WT	175	248	273
Relative transformation frequency ³¹	0	3–10	ND	1
Growth or transformation suppression ^{11–17}	+	–	ND	–
Inherited forms ²⁹		+		
Conformational alterations (antibody, hsc70 ^{33,34})	WT	mutant	(245–258) ND	WT
Half-life of protein ³¹	20 min	3–6 h	ND	7 h
Transactivation of a test gene ^{35,36}	+	–	ND	+

ND, not determined. WT, wild type.

16 or 18) binds to and promotes the proteolytic breakdown of p53 through the ubiquitin protease system⁴⁶. This holds for reticulocyte extracts, but needs to be confirmed for HPV-induced tumours and transformed cells. In degrading p53, however, E6 is causing a true loss of function, consistent with the role of p53 as a negative regulator of growth. The SV40 large T antigen and the adenovirus E1B 55K protein do not seem to act in this way.

There is, therefore, convergence of Rb and p53 proteins as the targets for the oncoproteins of DNA tumour viruses and of the Rb and p53 genes as the targets for mutation and selection during naturally occurring human or animal cancers.

How does p53 normally function?

Two hypothesis have been suggested to explain how p53 regulates passage through the cell cycle. First, p53 could regulate the assembly or function of the DNA replication-initiation complex^{47,48}. The p53 protein binds to the SV40 large T antigen which is required for the initiation and propagation of SV40 DNA replication: T antigen binds to the origin of DNA replication on the SV40 chromosome and acts as a helicase to promote DNA replication; it also binds the cellular α DNA polymerase required to synthesize SV40 DNA⁴⁸. Wild-type p53 protein, but not mutant forms, binds to large T antigen to give a complex that is neither competent to replicate SV40 DNA^{47,49} nor able to bind the α DNA polymerase⁴⁸. The p53 protein can inhibit the ATP-dependent helicase activity of T antigen⁴⁹. That all known p53 mutants, whether activated for transformation or from transformed cells, fail to bind to T antigen, even though they are selected for in cells lacking T antigen, suggests that p53 interacts with a cellular homologue of T antigen, that is, it initiates cellular DNA replication and entry into the S phase. The homologue and T antigen could share the same p53-binding site, thus explaining why p53 mutants do not bind to T antigen. In this way, p53 would negatively regulate entry into S phase. The p53 protein also associates with the viral DNA replication complexes in cells infected with herpesvirus⁵⁰. Perhaps p53 acts in the late G1 phase of the cell cycle to promote or prevent the assembly of a DNA replication-initiation complex.

Alternatively, p53 could act as a transactivator of gene transcription, either promoting or repressing messenger RNA synthesis. The p53 protein has a basic C-terminal region which is predicted to form an amphipathic helix-like structure, and it is probably this which is responsible for its binding to DNA⁵¹. The protein also has an acidic N-terminal region composed of the first 75 amino acids. If either this N-terminal region or the p53 protein is fused with a DNA-binding region of another protein (for example, the Gal-4 DNA-binding fragment), the resulting fusion protein will promote the transcription of a test gene regulated by the DNA-binding sequences^{35,36}. Mutant p53 for residue 135 or 215 fails to transactivate the test gene³⁵, whereas the p53 mutant for residue 273 retains this ability³⁶. These data indicate that some mutations that activate transformation affect gene transcription. Indeed, transformation-activating mutations alter the DNA-binding abilities of p53 (ref. 51). It now needs to be determined whether the p53 DNA-binding region recognizes a specific DNA sequence. Thus p53 could have a role in regulating gene transcription, perhaps of a set of genes that effect the passage from late G1 to S phase of the cell cycle.

Remaining questions

We need to know more about the frequency of p53 mutations in human tumours. Are there types of tumours which do not contain p53 mutations, and if so, why? The nature and position of a p53 mutation seems to be influenced by the cell or tissue type (Table 1). Is this due to differences in the environment of these cells (for example, mutagen exposure) or to selection of mutant alleles by different cancers? And we still do not know whether the point mutations in the p53 gene and the production of missense p53 proteins occur before the loss of the wild-type

allele. Does the heterozygous cell in a human with a mutant and wild-type allele have a growth advantage? If so, which p53 mutant alleles do this? Because individuals with Li-Fraumeni syndrome develop normally when heterozygous for mutant alleles for residues 245–258, it is unlikely that these alleles promote 'abnormal' growth in humans. How do each of the different mutant alleles (Table 2) affect the prognosis of individuals with cancers harbouring these mutants? Is there a correlation between the prognosis of a cancer and the presence or absence of a particular p53 mutation? How do tumours with wild-type p53 and Rb bypass the negative regulatory effects of these proteins? Such tumours could hold the key to understanding how the cell (or a DNA tumour virus) shuts off Rb and p53 function and moves from the G1 to the S phase.

And we still do not know the biochemical functions of Rb and p53. Are they involved with transcriptional regulation, DNA replication or both? What is the nature of the p53 gain of function? In this regard, cervical carcinomas could be particularly instructive. HPV type 16 and 18 are particularly common in these carcinomas, many of which express the E6 open reading frame. E6 could promote the degradation of p53 in these tumours⁴⁶, which would then be a clear example of a loss of function. In this case, there would be no reason to expect the selection of p53 gene mutations such as those in colon carcinomas. This would mean that cervical carcinomas associated with HPV would have a wild-type p53 gene, whereas cervical carcinomas without HPV would have mutant p53 genes; this is indeed the case⁵².

So far, several oncogenes and tumour suppressor genes have been identified that are in a mutant form in human cancers. The numbers of such genes known to us will continue to grow, and it is also possible that genes will be identified that alter cell growth when perturbed by completely different mechanisms (for example, by affecting repair functions, mutator genes, or chromatin and chromosome maintenance). Any one clone of cancer cells will be derived from some combination of these mutant genes. Determination of how these combinations disturb cellular growth control should enable us to understand the vast diversity of cancers. □

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ARTICLES

New use of BCG for recombinant vaccines

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BCG, a live attenuated tubercle bacillus, is the most widely used vaccine in the world and is also a useful vaccine vehicle for delivering protective antigens of multiple pathogens. Extrachromosomal and integrative expression vectors carrying the regulatory sequences for major BCG heat-shock proteins have been developed to allow expression of foreign antigens in BCG. These recombinant BCG strains can elicit long-lasting humoral and cellular immune responses to foreign antigens in mice.

VACCINES are the most cost-effective medical intervention known to prevent disease^{1,2}. Although two million children's lives are saved each year by immunization^{3,4}, it will be very difficult to protect more children in the poorest countries with existing technology. Significant problems inherent in existing vaccines include the need for repeated immunizations, reduced effectiveness due to maternal antibodies in children in the vulnerable first months of life, the need for injection, heat instability and cost. One strategy which might overcome some of these limitations is the development of recombinant vaccine vehicles which allow simultaneous expression of multiple protective antigens of different pathogens.

BCG (bacille Calmette-Guérin), a live attenuated bovine tubercle bacillus used to immunize against tuberculosis⁵, offers some unique advantages for developing such a multivaccine vehicle: (1) BCG is the most widely used vaccine in the world, having been given to over 2,500 million people since 1948, with a low incidence of serious complications (case fatality rate of 0.19/10⁶)⁶; (2) it can be given at or any time after birth, and is

unaffected by maternal antibodies; (3) BCG is given as a single inoculum and sensitizes to tuberculo-proteins for 5-50 years; (4) it is a potent adjuvant in experimental animals and man^{7,8}; (5) BCG can be administered as an oral vaccine; (6) it is the most heat stable of live vaccines; and (7) it is inexpensive to produce.

Multicopy and integrating shuttle vectors

First generation *Escherichia coli*-mycobacterial shuttle vectors, including temperate phages and plasmids, were used to demonstrate the feasibility of transforming and propagating recombinant DNA in *Mycobacterium smegmatis* and BCG^{9,10}. We found these first-generation vectors, for example plasmid pYUB12, to be unsuitable for the introduction and expression of foreign genes in mycobacteria because they were large, lacked useful unique restriction sites and, in the absence of antibiotic selection, were unstable in BCG. However, we have now identified a 1.8-kilobase (kb) segment (*oriM*) from plasmid pAL5000¹¹ which supports plasmid replication in *M. smegmatis* and BCG. Modification of *oriM* by polymerase chain reaction (PCR) mutagenesis was done to eliminate undesirable restriction sites and an *oriM* cassette was then used as the basis for developing second-generation extrachromosomal vectors using three additional PCR cassettes: (1) the *E. coli* plasmid replicon derived from pUC19 (*oriE*); (2) the Tn903-derived *aph* gene conferring kanamycin resistance (*kan*^r) as a selectable marker; (3) an expression cassette containing a mycobacterial promoter, a multiple cloning site and a transcriptional terminator. The resulting *E. coli*-mycobacteria shuttle vector (Fig. 1a) were shown to transform BCG at high efficiencies (10⁵-10⁶ c.f.u. per µg DNA) and maintain about five plasmid copies per genome equivalent (Fig. 1b).

An advantage of BCG is its innocuous persistence *in vivo* that could provide continuing immunization to recombinant antigen. However, the vectors carrying antigen genes need to be stably

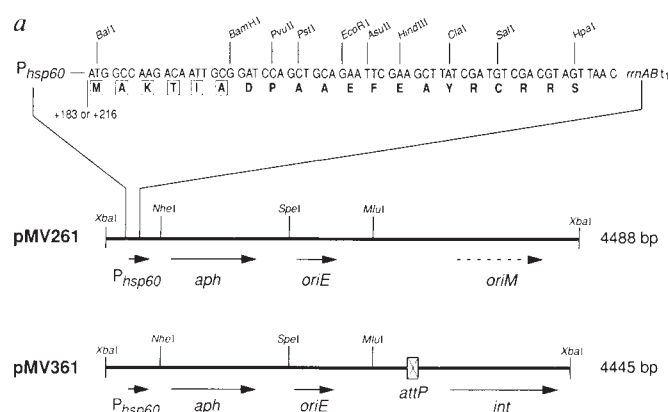
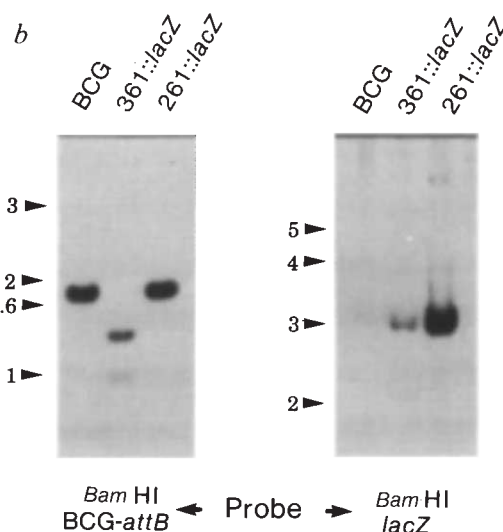


FIG. 1 *a*, BCG extrachromosomal (pMV261) and integrative (pMV361) expression vectors. Plasmids pMV261 and pMV361 share common elements including an expression cassette, the Tn903-derived *aph* gene conferring kanamycin-resistance as a selectable marker, and an *E. coli* origin of replication (*oriE*) derived from pUC19. They differ by the inclusion of either a mycobacterial plasmid origin of replication (*oriM*) or the *attP* and *int* genes of mycobacteriophage L5. The expression cassette contains 404 bp of the 5' regulatory region of the BCG *hsp60* gene³¹, including the first six amino acids (in boxes, single-letter amino-acid notation), 10 unique cloning sites and the *rrmAB11* transcriptional terminator³² aligned in the same transcriptional orientation as the other elements. *b*, Comparison of extrachromosomal (pMV261) and integration-proficient (pMV361) vectors. The *E. coli lacZ* gene was cloned into the unique *Bam*HI sites of pMV261 and pMV361 to form fusion proteins with *hsp60* and transformed into the Pasteur strain of BCG



as described previously¹⁰. Chromosomal DNAs were isolated from BCG and BCG transformants, digested with *Bam*HI, and Southern blot analyses were performed probing with either the BCG *attB* (ref. 13) gene or *lacZ*. Probing with *attB* (left panel) demonstrates that the pMV361::*lacZ* is integrated into the BCG chromosome as seen by the resolution of the *attB* sequence into an *attL* and *attR* fragments. Probing with the *lacZ* gene (right panel) demonstrates that pMV261::*lacZ* has a copy number of five as quantitated by an AMbis Direct Radioactivity Detector relative to the pMV361::*lacZ* site-specific integrant.

maintained *in vivo*. Selections for maintenance of plasmids *in vivo* which are useful in other bacterial systems¹² have not yet been adapted for the mycobacteria and as an alternative approach we have exploited the site-specific integration system of a temperate mycobacteriophage¹³. The integration-proficient vector, pMV361, was constructed by replacing the *oriM* region of pMV261 with a DNA segment carrying the attachment site (*attP*) and the integrase (*int*) gene (Fig. 1*a*) from the mycobacteriophage L5¹³. BCG is transformed with pMV361 through a site-specific integration into the chromosomal *attB* site with a similar efficiency to that of extrachromosomal vectors. Because the phage *xis* gene is not present the integrated vectors are stably maintained even without antibiotic selection¹³. Compatibility with *oriM*-containing plasmids also adds flexibility to these vector systems.

Foreign antigen expression in BCG

Although little is known about mycobacterial gene expression, particularly in the intracellular environment of the host, the abundant and highly conserved *hsp60* and *hsp70* cognate stress proteins are major antigens of mycobacteria, rickettsia, coxiella and chlamydia¹⁴⁻¹⁸. The Hsp60 and Hsp70 proteins are essential under all growth conditions, but are expressed at higher amounts in response to stress¹⁹. Thus the regulatory sequences of the BCG *hsp60* and *hsp70* genes were chosen to drive the expression of foreign antigen genes in BCG. Primer extension analysis identified two shock-responsive transcription start sites for the *hsp60* (Fig. 2*a*) and *hsp70* (data not shown) genes which had not been characterized previously. Endogenous BCG Hsp60 and Hsp70 protein levels also increased in response to stress with heat and acid but not peroxide (Fig. 2*b*).

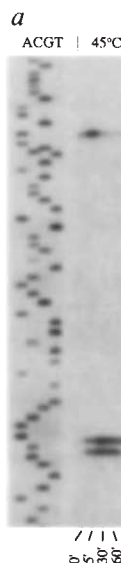
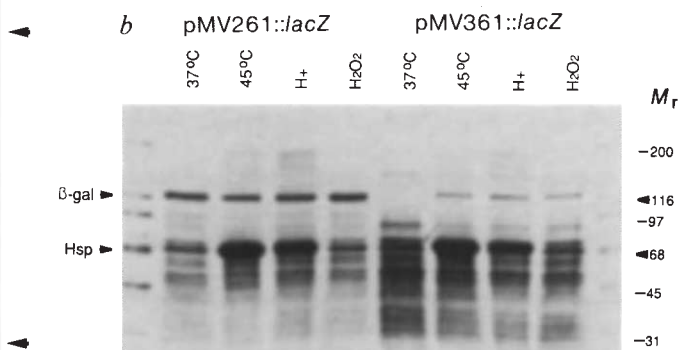


FIG. 2 *a*, Primer extension analysis of *hsp60* transcription. BCG was grown to mid log-phase (Absorbance at 650 nm, 0.44) in Dubos broth (Difco) supplemented with albumin dextrose complex (D-ADC broth) at 37 °C then rapidly shifted to 45 °C. Total RNA was extracted from culture aliquots taken at 0, 5, 30, 60, 120, and 180 min after heat-shock and subjected to primer extension³³ using the 5'-CTGCCACTCGCTGCGACG-3'. Two transcription start sites were identified for the BCG *hsp60* gene at positions 183 and 216 bp (arrows) upstream of the ATG translational start site of *hsp60* protein. As shown, messenger RNA originating at the two transcription start sites markedly increased with heat shock, but differed with respect to both the presence of mRNA at 37 °C and the maintenance of the elevated level at 45 °C. *b*, β -galactosidase expression by extrachromosomal and integrative BCG recombinants. BCG containing pMV261::*lacZ* or pMV361::*lacZ* cultures were grown to mid log-phase at 37 °C and each was divided into four aliquots. Three samples from each culture were incubated at 37 °C for an additional 30 min, with two of the samples being subjected to stress by the adjustment to 0.002% H₂O₂ or pH 5 with the addition of H₂PO₄. Another sample from each set was stressed by incubation at 45 °C and incubated for an additional 30 min at either 45 °C or 37 °C and adding H₂O₂ or H₂PO₄. Cells were collected and sonicated, and the lysates were subjected to 4–15%



gradient SDS-PAGE, fixed and stained with Coomassie blue. Numbers in right margin denote relative molecular mass (*M_r* in kDa). Arrows, β -gal and the *hsp60*–*hsp70* doublet at about 65 and 71 kDa, respectively.

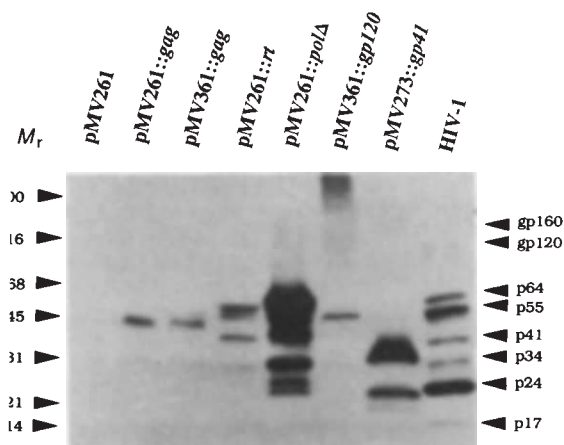


FIG. 3 Expression of HIV-1 antigens (IIIB isolate) in recombinant BCG. Genes encoding HIV-1 *gag*, *rt* (reverse transcriptase), *polΔ* (polymerase minus protease), *gp120*, and *gp41* proteins were amplified by PCR, fused to the sixth codon of the *hsp60* gene in pMV261 or pMV361, or the HSP70 gene³⁷ in pMV273 and transformed into BCG. Lysates of recombinant BCG were analysed for expression by western blot analysis of 4–20% gradient SDS-PAGE using human sera from a patient infected with HIV-1 and ¹²⁵I-labelled *Staphylococcus aureus* protein A. Arrows in left margin denote *M_r* standards. Arrows in right margin indicate apparent *M_r* (in kDa) of HIV-1 proteins.

The *E. coli lacZ* genes was placed under control of the BCG *hsp60* regulatory sequences to evaluate its potential for expressing foreign genes. Striking constitutive expression of the *hsp60* driven *lacZ* gene, in excess of 10% of total BCG protein, was observed from the extrachromosomal vector (Fig. 2b, pMV261::*lacZ*). Expression of *hsp60*::*lacZ* from the integrative vector was lower than that from the extrachromosomal vector; however, expression from the integrative vector, in contrast to the expression from the extrachromosomal vector, showed a coincident increase in Hsp60-β-gal in response to stress with heat, acid, and peroxide (Fig. 2b).

Antigen genes from a variety of pathogens including viral, parasitic and bacterial pathogens were cloned and expressed in recombinant BCG (rBCG). Several of the antigens of human immunodeficiency virus type 1 (HIV-1), which are relatively difficult to express at high amounts in other bacterial systems, were expressed in rBCG. Although expression of the HIV-1 *pol* coding region (minus protease) was substantial, expression

quantities for most of the *hsp60*-driven HIV-1 antigen genes did not rival those of *hsp60*-β-gal (Fig. 3). Extrachromosomal expression sometimes appeared to be lethal and it was only possible to express foreign genes in the single-copy integrative vectors (for example HIV-1 gp120). In other cases extrachromosomal and integrative expression were comparable (for example HIV-1 *gag*, Fig. 3). These studies establish the value of the BCG *hsp* regulatory sequences in driving expression of foreign genes in mycobacteria and suggest that homologous *hsp* sequences may be generally useful for enhancing expression of foreign genes in other recombinant vaccines.

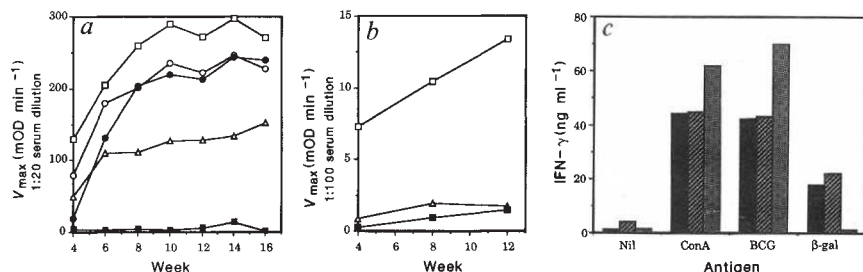
Immune responses to model antigens

To investigate the immunological capabilities and possible limitations of rBCG vaccines, we examined the humoral and cellular immune responses to several antigens expressed in BCG. Mice (BALB/c) were immunized with a rBCG expressing β-gal on a plasmid as a fusion protein with the first six amino acids of BCG *hsp60* antigen. Antibodies to β-gal were found in mice inoculated with the rBCG by all routes (Fig. 4a). Most striking was that very low numbers of live rBCG (as few as 200 c.f.u. given intravenously, and 10⁴ c.f.u. intradermally) were sufficient for high levels of antibody to β-gal (Fig. 4a). After a delay, antibody levels were sustained for many weeks. Antibodies to β-gal were not only higher (ELISA titre of 1:30,000, Fig. 4a) than responses to BCG sonicate (titre 1:5,000), but persisted much longer than those to BCG, which peaked only 2–4 weeks after immunization and waned thereafter. Thus greater antibody responses may be achieved to recombinant proteins expressed by rBCG than to antigens of BCG itself.

The growth of rBCG and the stability of the extrachromosomal plasmid without *in vivo* drug selection were established by the recovery of rBCG from mouse spleens 2–4 weeks after immunization. All BCG colonies were kan^r and expressed β-gal, and the number of bacilli recovered was >30 times that estimated to be localized in the spleen from the original inoculum. Viable rBCG recovered from spleen diminished between 2 and 4 weeks which is similar to non-recombinant BCG, but all rBCG recovered remained kan^r and expressed β-gal. These results, coupled with the delayed but sustained anti-β-gal antibody responses, establish that *hsp60*-driven expression of *lacZ* was effective for the expression of this model antigen *in vivo*, and suggest that actively growing rBCG can provide continuous boosting to the foreign antigen from a single immunization.

To assess the humoral immune response to an antigen expressed at substantially lower levels than β-gal in BCG, mice

FIG. 4 a, Antibody production to β-galactosidase in mice immunized with rBCG::*lacZ*. The rBCG was grown to mid log-phase in Dubos-ADC broth, then concentrated 20-fold by centrifugation. The concentrated bacteria were resuspended in PBS + 0.05% Tween 80 and cup sonicated briefly to disperse clumped bacteria. Six-week-old BALB/c mice were inoculated with a single dose of 2 × 10², 2 × 10⁴ or 2 × 10⁶ c.f.u. (determined after inoculation in each experiment) by either intravenous (i.v.), intradermal (i.d.), or intraperitoneal (i.p.) routes. Sera were obtained biweekly, pooled and analysed by



a β-gal-specific enzyme-linked immunosorbent assay (ELISA) with horse radish peroxidase-conjugated goat anti-mouse immunoglobulin and ABTS colour developing reagent (Kirkegaard and Perry). Colour reactions were read kinetically over 3 min on a *V_{max}* (maximum reaction velocity) plate reader (Molecular Devices) and results are presented as *V_{max}* rates (MOD min⁻¹). *V_{max}* rates of 200 MOD min⁻¹ and 300 MOD min⁻¹ were equivalent to standard ELISA titre determinations of about 1:10,000 and 1:30,000, respectively. □, 10⁶ i.v.; ○, 10⁶ i.p.; △, 10⁴ i.d.; ●, 10² i.v.; ■, naive. b, Antibody production to tetanus toxin fragment C in mice immunized with rBCG::*tox*C. DNA encoding fragment C of *Clostridium tetani* toxin (*tox*C) was cloned and fused in frame with the sixth codon of BCG *hsp60* into pMV261. NIH Swiss mice were inoculated with predetermined c.f.u. by the

indicated routes. Parallel inoculations were made with the same preparations that had been heat killed for 30 min at 70 °C. Sera were obtained monthly, pooled and analysed at 1:100 dilution by ELISA as above using plates coated with 10 μg ml⁻¹ tetanus toxoid (Connaught Labs). □, Live 10⁶ i.d.; △, dead 10⁶ i.d.; ■, naive. c, INF-γ production by lymphocytes from mice immunized with rBCG::*lacZ* and rBCG::*gp120*. Splenocytes (ACK-treated 5 × 10⁶ ml⁻¹, where ACK is ammonium chloride/potassium carbonate) were cultured in 0.5 ml 50% RPMI 1640 medium, 50% EHAA medium (Gibco), 10% FCS supplemented with: media (Nil); concanavalin A, 5 μg ml⁻¹; WHO BCG Vaccine, 100 μl ml⁻¹ culture; or β-galactosidase, 10 μg ml⁻¹. IFN-γ levels in supernates from 72 h cultures were determined by ELISA³⁴. Solid bars, 10² rBCG *lac* (i.v.); hatched bars, 10⁶ rBCG::*lacZ* (i.p.); dotted bars, rBCG::*gp120* (i.p.).

were immunized with rBCG expressing the fragment C (ToxC) of tetanus toxin²⁰. This fragment (relative molecular mass, 50,000) lacks toxic activity, but binds to the cellular glycolipid receptor. ToxC expression was 1/250th that of β -gal on a weight basis (1/100 on a molar basis). Immunization with a single dose of rBCG::toxC led to antibody levels that increased for at least 12 weeks (Fig. 4b). The ability of rBCG to induce antibodies is therefore not unique to β -gal or to antigens expressed only at high levels. Heat-killed BCG::toxC was not effective at immunization, supporting the view that the persisting immune responses with rBCG are due to growth and persistence of the vaccine. That IgG responses were obtained implies that T cells also responded to the rBCG. This was confirmed by assessment of cellular immune responses. Lymphokines, specifically interferon- γ (IFN- γ), were produced *in vitro* by spleen cells stimulated with β -gal obtained only from animals inoculated with rBCG::lacZ, and not with rBCG::gp120, (Fig. 4b).

Cytotoxic T lymphocytes (CTL) are critical for resistance to many viral and bacterial infections²¹. There was much cytotoxic activity in splenocytes from mice immunized with 2×10^6 , or as few as 200 c.f.u. rBCG::lacZ given 19 weeks earlier (Fig. 5a-c). To learn whether the induction of CTL precursors is likely to be a general property of rBCG vaccines or whether it is peculiar only to β -gal or to antigens expressed at very high levels, lymphocytes from mice immunized with rBCG expressing HIV-1 gp120 antigen from pMV361 were examined for cytotoxic activity against P815 target cells pulsed with the P18 peptide. This peptide is the major CTL epitope of HIV gp120 in BALB/c mice and killing has been shown to be restricted to the major histocompatibility complex (MHC) class I D^d allele²². There was notable specific lysis (Fig. 5d), confirming the induction of CTL to the gp120 peptide. The failure to detect killing of the target cells in the absence of the peptide and the fact that our P815 cells did not express MHC class II antigens, suggest that lysis is mediated by MHC class I-restricted CTL.

Discussion

The ultimate goal in the development of vaccines is to induce sustained immunity against multiple pathogens with the minimum number of inoculations. Whether any single vehicle or delivery system can effectively evoke the required immune responses to a wide variety of antigens in a single dose remains to be seen. Nevertheless, it has been possible to express, in addition to the three antigens described here, antigens from 17 viral, bacterial and parasitic pathogens in rBCG. All the immunological data presented here derive from single-dose immunizations. Although notable amounts of antibodies were produced and maintained to both β -gal and to ToxC, the lack of significant titres (>1:100 dilution) of antibodies found to HIV gp120 suggests that the level of expression may be important. In the case of both β -gal and ToxC, antibodies were induced by the intradermal route used for human immunization. It is not evident how an intracellular organism whose recombinant protein would presumably be subjected to proteolysis within the lysosomal compartment of macrophages, can effectively elicit antibody responses, which generally recognize epitopes on intact antigens. Because under standard conditions for culture of BCG vaccine substrains only 25–40% of acid-fast bacilli are able to form colonies²³, the rBCG vaccines must contain nonviable organisms capable of releasing relatively intact recombinant antigens recognized by B cells. Nevertheless, killed rBCG expressing ToxC failed to immunize, indicating that dead bacilli are not effective immunogens. This result and the finding that rBCG expressing β -gal have been isolated at least 4 weeks after immunization, support our interpretation that the continuing production of antibodies is due to antigen molecules being released from the rBCG over long periods of time *in vivo*.

Because mycobacteria are rapidly phagocytosed and contained within the endosomal compartment of phagocytic

cells^{24,25}, it was not unexpected that rBCG would be an effective vehicle for inducing both T-helper cells capable of inducing B-cell production of IgG antibodies and the T cells capable of secreting lymphokines such as IFN- γ (Fig. 4b). It was not obvious, however, that it would be effective in stimulating cytotoxic T lymphocytes. That BCG can generate CTL restricted by MHC class I in mice^{26,27} encouraged us to assay for cytotoxic activity in animals immunized with rBCG. CTL were generated to both β -gal and HIV-1 gp 120 (Fig. 5). There are two possible interpretations: some rBCG may escape from lysosomes and penetrate into the cytoplasmic compartment; or because the general examples of antigen processing come mainly from studies of antigens that persist for only short times in antigen-presenting cells, conceivably antigens produced by rBCG persisting for long times in the endosomal compartment can be selected and presented to T cells both by MHC class I and class II antigens.

Although the immune responses obtained to recombinant antigens expressed in BCG reported here are encouraging, much remains to be learned before rBCG can be perfected as a multivaccine vehicle. Conditions may have to be adjusted to allow optimal expression of different antigens. For some it may be important to include signal sequences that enable recombinant proteins to be secreted intact from the rBCG. New formulations for maximizing the effectiveness of rBCG given as an oral vaccine remain to be developed. A major challenge will

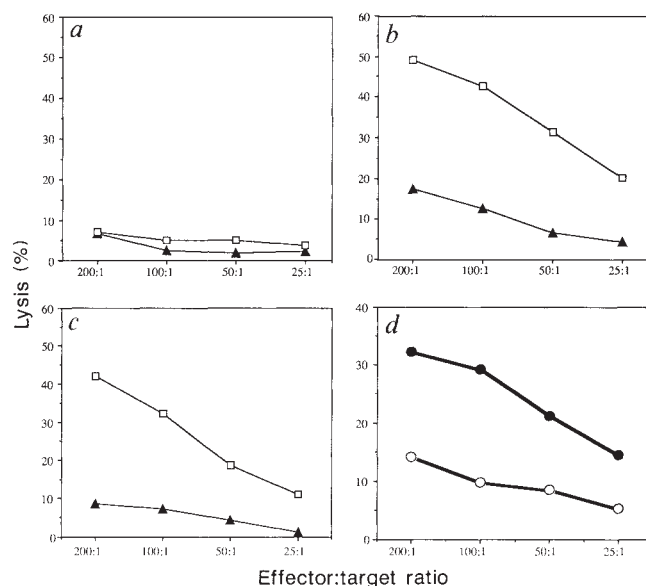


FIG. 5 Cytotoxic T-lymphocyte responses to recombinant antigens expressed in rBCG. a–c, Specific cytotoxicity to target cells expressing β -galactosidase. BALB/c mice were immunized as indicated. Splenocytes (ACK-treated, $5 \times 10^6 \text{ ml}^{-1}$) were stimulated *in vitro* in 10 ml in upright T25 flasks by coculture for 5 days with mitomycin C-treated cells transfected with the lacZ gene (C3–4 cells)³⁵ ($5 \times 10^5 \text{ ml}^{-1}$). Then a 4 h ^{51}Cr -release assay was done using P815 (▲) and P13.1 cells³⁶ (P815 cells transfected with the lacZ gene) (□) as targets. Effector:target ratios are as indicated using 5,000 targets per well. Specific lysis was calculated as follows: % specific lysis = $100 \times (\text{release by effector cells} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. Spontaneous release in the absence of effector cells was less than 20% of maximal release by 10% SDS in all cases. a, β -gal-IFA ($10 \mu\text{g i.p.}$); b, rBCG::lacZ (2×10^6 c.f.u. i.p.); c, rBCG::lacZ (2×10^2 c.f.u. i.v.). d, Specific cytotoxicity to the HIV-1 envelope immunodominant CTL epitope (P18 peptide). BALB/c mice were immunized with 10^6 c.f.u. rBCG containing the integrating vector expressing gp 120 (pMV361::gp 120). Splenocytes were cocultured as above with mitomycin C-treated P815 cells ($5 \times 10^5 \text{ ml}^{-1}$) that were pulsed with $250 \mu\text{g ml}^{-1}$ of P18 peptide for 1 h. After incubation for 5 days, cells were assayed for killing by 4 h ^{51}Cr -release as above using P815 targets with (●) or without (○) pulsing for 1 h with $250 \mu\text{g ml}^{-1}$ P18 peptide.

be to immunize against antigens, particularly viral antigens, whose protective epitopes require conformations or modification, such as glycosylation and assembly, that cannot be acquired in most procaryotes. There is a precedent in viral systems, however, that priming for immunological memory at the level of the T-helper cell, as by immunization with influenza virus internal antigens, is sufficient to allow induction of accelerated antibody responses to the surface antigens upon infection with virus²⁸⁻³⁰. Thus, even if viral antigens expressed in rBCG fail to induce virus-neutralizing antibodies, immunological

memory may be generated such that an efficient secondary response would be obtained following natural infection by virus. This would be particularly important as BCG, which is unaffected by maternal antibodies, can be given earlier in life than most viral vaccines. The hope would be that T-cell memory can ensure protection against severe morbidity and death. We believe these results establish the molecular and immunological foundation for a novel live-vaccine vehicle that may prove useful in stimulating both humoral and cellular immune responses to a wide variety of viral, bacterial and protozoal antigens. □

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LETTERS TO NATURE

Giant tunnelling anisotropy in the high- T_c superconductor $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$

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TUNNELLING spectroscopy has been one of the most fruitful methods in the study of superconductors^{1,2}. Excellent agreement has been obtained between theory and experiment, and even the fine details of the tunnelling spectra for conventional, low-transition-temperature (low- T_c) superconductors have been explained in terms of electron-phonon interactions. The low- T_c materials are generally isotropic enough for accurate measurements to be made on polycrystalline specimens; in contrast, it has been difficult to obtain reliable and reproducible tunnelling data for the highly anisotropic high- T_c materials³. We have overcome these difficulties by performing break-junction tunnelling measurements⁴ on extremely thin single crystals, and show here that the tunnelling spectra of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ are indeed highly anisotropic. In the superconducting state, for electrons tunnelling parallel to the copper oxide planes, there are no electronic states at the Fermi level. In the normal state the tunnelling conductance is nearly independent of the d.c. bias voltage. Tunnelling perpendicular to the copper oxide planes was found to be qualitatively different from that parallel to the planes, and we suggest that electron scattering processes play an important role here.

In its most ideal realization, an electron tunnelling measurement provides us with the electronic density of states (DOS)¹. In particular, for a superconductor-insulator-superconductor junction, one can directly measure the energy gap 2Δ by observing a sudden increase of conductivity at bias voltages, V , exceeding $2\Delta/e$ (here e is the electron charge). The bias-voltage-dependent tunnelling conductance (the 'tunnelling spectrum') is related to the energy-dependent DOS by a simple integral¹. At low temperatures the conductivity of the junction is close to zero for $V < 2\Delta/e$, indicating zero DOS. For most of the low- T_c superconductors the experiments are very well described by BCS theory⁵. The copper-oxide-based high- T_c superconductors, however, did not exhibit the expected zero conductance^{5,7}. One is forced to assume either that BCS theory is not valid for these materials, or that the electron transport cannot be described as an ideal tunnelling process. Is it possible, for example, that the tunnelling is strongly influenced by inelastic electron scattering? Complex tunnelling conductance curves and 'zero-bias anomalies' have been seen in junctions with magnetic impurities⁸⁻¹³. The well known structural anisotropy of the copper-oxide-based high- T_c superconductors may also result in nontrivial effects. The tunnelling process is inherently anisotropic, because the particles with non-perpendicular incidence angle are less likely to travel through the potential barrier. What happens if the Fermi surface of the material is so anisotropic that there are no electron momenta perpendicular to the junction surface?

We attempted to answer these questions by performing tunnelling measurements on break junctions⁴ fabricated at low temperature, in vacuum. By using ultra-thin single crystals of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$, we were able to obtain reproducible superconductor-insulator-superconductor tunnelling spectra in high- T_c superconductors. We found that parallel and perpendicular to

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the CuO planes, the tunnelling and its temperature dependence are very different. For 'in plane' tunnelling, a gap structure is visible at low temperatures, with the tunnelling conductance vanishing at zero bias. At temperatures above T_c , the conductance is nearly independent of the voltage. For 'perpendicular' tunnelling, conductance is always nonzero, and its temperature dependence is well approximated by a smooth function, with no reference to T_c .

For the tunnelling measurements in the ' a - b plane', we first cleaved a strip $\sim 1,000$ Å thick and 100 μm wide from a single crystal of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ (ref. 14). We mounted the ultra-thin specimen on a crystal of $\text{Bi}_2\text{Sr}_2\text{YCu}_2\text{O}_8$ (a high-resistance semiconductor). Electrical contacts to gold wires were made by silver epoxy. After the heat treatment the contact resistance was ~ 1 Ω; the four-probe room-temperature resistance of the sample varied between 20 and 300 Ω depending on the sample thickness and contact distance. The $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8/\text{Bi}_2\text{Sr}_2\text{YCu}_2\text{O}_8$ assembly was glued to an elastic strip of metal and fixed on the cold finger of a helium-flow cryostat. A precision-mounted screw pushed the back of the metal strip. When the temperature reached ~ 10 K, a 'break junction' was produced by bending the metal strip with the screw.

For ' c -axis' tunnelling, we first prepared electrical contacts on the opposite, cleaved, surfaces of a crystal. We glued the sample to the inside of a U-shaped elastic strip so that the electrical contacts faced the two arms of the U. We cooled the device in vacuum and observed typical c -axis conductance: resistivity increasing with decreasing temperature, turning to superconductivity at T_c . At low temperature, the crystal was cleaved by increasing the separation between the arms of the U with the screw mechanism. The break junction was formed between the freshly cleaved surfaces. We also prepared junctions in air at room temperature. The c -axis tunnelling curves did not depend on the preparation conditions. The a - b plane tunnelling seems to be sensitive to the exposure to air, and good junctions were obtained only by breaking the sample in vacuum.

Several a - b plane and c -axis tunnelling curves are shown in Fig. 1 (upper and lower panels, respectively). All of these measurements were taken at low temperature ($T \ll T_c$). The

junction resistances are in the range $R = 500$ Ω–500 kΩ, much larger than the contact resistance or sample resistance. In both cases a supercurrent is visible at zero bias, but only for the junctions with lower resistance. This current was suppressed by external magnetic field of ~ 1 kG. We assume that this current is due to the Josephson effect, although we did not observe the interference pattern typical of Josephson current in evaporated junctions with well defined geometry and uniform current distribution¹.

The temperature dependence of the tunnelling is illustrated in Fig. 2. For each temperature the conductivity is normalized to the arbitrary, but fixed (and relatively high) value at $v=200$ mV. This was necessary to correct for the thermal expansion effects. (The normalization constant, $G(200$ mV), is nearly independent of the temperature up to 80 K.)

We want to call attention to several important features of the spectra presented in Figs 1 and 2. At low temperatures the zero-bias conductance is zero for tunnelling parallel to the a - b plane (if the pair-tunnelling contribution is subtracted), whereas it is finite for c -axis tunnelling. The a - b plane conductance exhibits a gap-like structure near $V = 55$ mV, but no well-defined structure is seen in the c direction. The a - b plane spectra become relatively smooth above T_c , whereas the c -axis curves continue to show temperature dependence. In fact, the c -axis tunnelling curves can be approximately fitted by an empirical formula, $G = G_0 \log(\xi/\xi_0)$, where $\xi = (eV)^2 + (\alpha k_B T)^2$ and G_0 , α and ξ_0 are fitting parameters. Finally, at large bias the a - b plane tunnelling conductance decreases, but the c -axis conductance increases with increasing bias voltage.

In the superconducting state the a - b plane tunnelling is best understood in terms of a strong, temperature-dependent variation of the DOS, although fitting the data to the weak coupling BCS theory is clearly out of question (even if the gap $2\Delta = 3.5k_B T_c$ is scaled to a higher value). Introducing finite lifetime for the Cooper pairs (as done by Dynes *et al.*¹⁵) does not help, either. Strong coupling calculations, like the one presented by Allen and Rainer¹⁶, are being tested. The vanishing tunnelling conductance at zero bias unambiguously shows that at low temperatures the DOS at the Fermi level is zero. Thus the tunnelling results confirm an important aspect of the BCS theory. There is, however, no evidence for a fully developed BCS gap; the data indicate a nonzero DOS at energies different from the Fermi energy. Above the critical temperature the tunnelling conductance does not exhibit the 'V'-shaped curves seen for evaporated junctions⁷, but it is similar to the point-contact measurements of Huang *et al.*¹⁷.

To evaluate the c -axis tunnelling results, we recall that the tunnelling conductivity can be expressed in terms of an integral containing the tunnelling probability, the density of states and the Fermi function. In a somewhat simplified way, the shape of the low-temperature curves for c -axis tunnelling can be attributed either to the DOS having a strong energy dependence near the Fermi energy, or to scattering effects, introducing a nontrivial energy dependence into the transmission probability. Tunnelling to systems with localized electronic states, like amorphous $\text{Ge}_{1-x}\text{Au}_x$ (ref. 8), or amorphous Ge (ref. 9), belong to the first category. Junctions with magnetic impurities are good examples of the second category. Kondo-type scattering, observed for Cr-doped¹⁰ and O_2 -doped junctions¹¹, and discussed by Mezei and Zawadowski¹², introduces a logarithmic voltage and temperature dependence into the transmission probability, resulting in spectra similar to ours. Kirtley and Scalapino¹³ recently obtained 'V'-shaped tunnelling spectra by considering inelastic scattering processes.

The first attempt to measure anisotropic tunnelling on high- T_c single crystals was by Kirtley *et al.*¹⁸. These authors, as well as Tsai *et al.*¹⁹, concluded that in $\text{YBa}_2\text{Cu}_3\text{O}_7$ the gap is likely to be anisotropic. Ekino and Akamitsu²⁰ found a smaller gap in the c -axis configuration than in the a - b plane for $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$. A similar conclusion was reached by Briceno

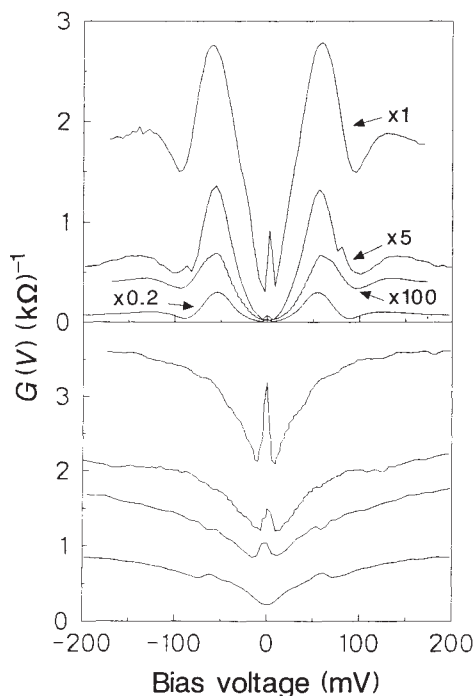


FIG. 1 Differential conductance, G , as a function of bias voltage, V , obtained from several break junctions at $T=9$ K. Top, a - b plane; bottom, c -axis configuration.

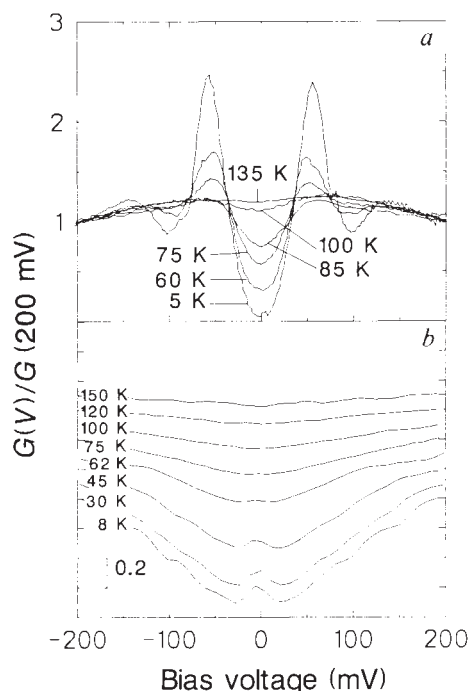


FIG. 2 Tunnelling conductance *a*, for *a*-*b* plane tunnelling; *b*, for *c*-axis tunnelling in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ for the temperatures indicated. The curves are normalized to the conductance at a bias voltage of $V = 200$ mV. Successive *c*-axis curves are shifted by 0.1 unit for clarity. All data in this figure were obtained on junctions prepared in vacuum at low temperature.

and Zettl²¹. Most of these measurements were made with a metallic tip in a point-contact configuration, where the direction of tunnelling is not very well defined, as the contact can actually penetrate the specimen^{22,23}. In contrast, our study (Fig. 1) illustrates that although there are variations between tunnelling spectra belonging to the same class (*a*-*b* plane or *c* axis), the difference between the two classes is much more marked.

We should point out that in a point-contact or break-junction geometry, variations in the tunnelling spectra can arise because of the variations in the local DOS²⁴, as shown by tunnelling microscope studies^{25,26}. In fact the sample may cleave preferentially between the BiO layers, and the *c*-axis tunnelling curves could measure the ' BiO density of states'. For our measurements, the total absence of the 0.1-eV insulating energy gap, seen by Hasegawa and Kitazawa²⁵ in the BiO layers, makes this unlikely.

We conclude that the tunnelling spectra parallel and perpendicular to the copper oxide planes are determined by entirely different microscopic parameters or microscopic mechanisms. The survey of the literature and our experience indicate that it is hard to realize pure *a*-*b* plane or *c*-axis tunnelling. In the strongly anisotropic copper-oxide-based superconductors it will be extremely difficult, if not impossible, to perform something similar to the 'classic' McMillan-Rowell evaluation of the tunnelling spectra^{1,2} where variations of a few per cent in the tunnelling conductivity are attributed to electron interactions. This type of evaluation may have better chances for the cubic $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ material²⁷. □

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Structure and bonding in alkali-metal-doped C_{60}

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It has been shown recently that M_xC_{60} (M = alkali metal) is metallic at 300 K for some M and x (ref. 1) and superconducts below 18 K for M = potassium². These observations give further impetus to studies of the molecular³ and solid-state⁴ properties of fullerenes. Here we report on X-ray diffraction studies of the structure and bonding in alkali-metal-doped solid C_{60} (fullerite). Powder diffraction data obtained from equilibrium compositions of C_{60} doped to saturation with K or Cs show that the face-centred cubic lattice of pure C_{60} (refs 5-7) transforms to body-centred cubic at these doping levels, with a saturation composition close to M_6C_{60} . The rotational disorder of the fullerene molecules in pure C_{60} at 300 K is absent in the fully doped compounds.

Because our samples may be different from those of refs 1 and 2, a definite identification of the superconducting phase cannot be made at this time. No information was given on chemical composition or X-ray structure of the latter samples, and the conductivity and superconductivity measurements were performed mostly on nonequilibrium compositions. Both the normal conductivity and superconductivity were ascribed to the formation of a doped molecular crystal, analogous to 'ionic salt' intercalation compounds in which the host lattice and its electron states are largely unperturbed by the guest, and the metallic properties originate in delocalization (or intermolecular hopping) of transferred electrons. This implies that metallic character will be optimal at a composition K_3C_{60} , in which one delocalized electron per K leads to a half-filled conduction band derived from a threefold-degenerate molecular level^{1,2}. This suggestion was justified by the observation that the 300-K conductivity first increases and then decreases with time of exposure to potassium vapour, on a timescale of about 4 h when the vapour temperature is held at about 75 °C. It was further suggested that metal atoms occupy vacant tetrahedral and octahedral sites in the f.c.c. lattice¹, thus maintaining the same close packing and intermolecular contact as in pure C_{60} (refs 5-7).

Our results support some features of such an intercalation analogy, but the equilibrium structural modifications on doping

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to saturation are more profound than envisaged. We measured chemical composition, weight uptake and X-ray diffraction patterns for C_{60} powders vapour-doped with K or Cs in evacuated sealed tubes. Reaction temperatures were in the range 200–225 °C, for times ranging from 12 hours to 7 days. A gradient of 2–5 °C was maintained along the tube to prevent metal from condensing onto the C_{60} . X-ray profiles show a single, highly crystalline doped phase in all reactions performed so far. They also strongly suggest that this is the only equilibrium structure under these reaction conditions—the profile after a 12-h, 200 °C exposure to potassium revealed a mixture of C_{60} and the doped phase. Weight uptake of pure C_{60} on doping to saturation with potassium indicates a K/ C_{60} ratio of 6.7, consistent, to within experimental error, with the ratio of 7.3 obtained from potassium elemental analysis. Weight uptake of a K-doped sample made from unpurified toluene extract (~20% C_{70}) gave a slightly larger ratio. An attempt to measure flotation density in halogenated hydrocarbons gave ambiguous results because of the occurrence of chemical reactions, as indicated by colour changes; this otherwise null result supports the notion that these are soluble ionic-salt compounds.

Figure 1 shows 300-K powder diffractions, measured on beam-line X10-B at the Brookhaven National Synchrotron Light Source (wavelength 0.9617 Å), from 0.5-mm capillaries of >99.9% pure C_{60} doped to saturation with K (Fig. 1a) and Cs (Fig. 1b). All reflections from both compounds can be indexed on body-centred cubic lattices, with lattice parameters $a = 11.39$ and 11.79 Å respectively. Saturation doping therefore induces a significant rearrangement of C_{60} molecules with respect to the pristine f.c.c. phase^{5–7}. The peak widths are not resolution-limited; coherence lengths inferred from the Scherrer formula are 500 and 450 Å for K-doped and Cs-doped profiles respectively, about one third of the value for the starting f.c.c. powder. We confirmed that the broad diffuse peak near 1.8 Å is due to scattering from the glass capillaries; there is no evidence for an amorphous doped phase. The details of Rietveld refinements of these profiles will be presented in detail elsewhere. We summarize here the salient features of the Cs-doped structure, for which an integrated intensity R factor of 4.3% is obtained with 33

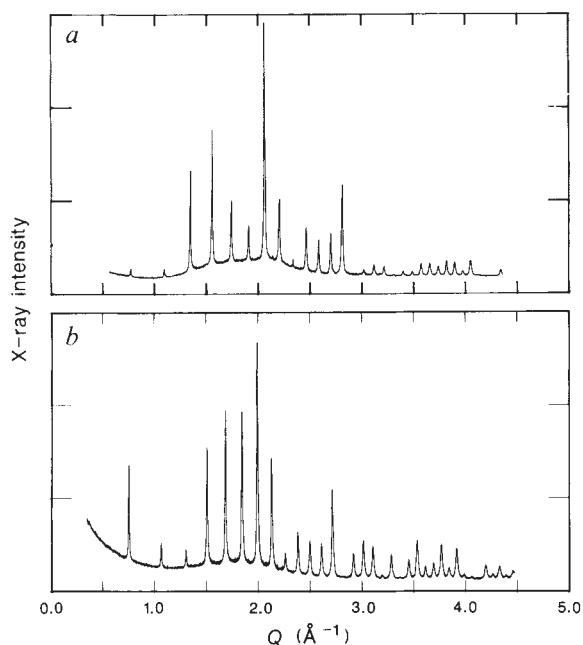


FIG. 1 X-ray powder diffraction profiles of K-doped (a) and Cs-doped (b) C_{60} . Intensity is plotted on a linear scale as a function of $Q = 2\pi/d = 4\pi \sin \theta/\lambda$; the data were recorded with 2θ steps of 0.01° or 0.015° for 2–4 s per point, which gave about 100,000 total counts at the strongest peak. These figures show raw data, including scattering from air and the glass capillaries.

peaks. The chosen space group is $Im\bar{3}$, and a refinement with all C–C distances constrained to be equal gives a value 1.44 Å for this distance. With this constraint there are only two variable positional parameters, one each for C and Cs, and the corresponding coordinates are: C1 at 0.0611(1), 0.0, 0.2968(2); C2 at 0.1222(1), 0.0989(1), 0.2590(1); C3 at 0.0611(1), 0.1978(1), 0.2212(1); Cs at 0.0, 0.5, 0.2781(1). (Numbers in parentheses represent estimated standard deviations.) The R factor is not improved significantly by allowing two different bond lengths in the hexagons (C1–C2 and C2–C3 in Fig. 2), or by an unconstrained refinement of all eight C positional parameters. Isotropic Debye–Waller factors yielded mean-square thermal amplitudes of 0.018 and 0.039 Å² for C and Cs respectively. The K-doped structure is essentially the same, apart from the value of a .

Figure 2 shows a schematic view of a cube face, from which the essential features of the composite structure may be appreciated. Two equivalent C_{60} molecules per cell are centred at (0, 0, 0) and $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ (the latter is omitted from Fig. 2 for clarity). The C atom positions are localized to within a mean thermal displacement of 0.13 Å, indicating that orientational disorder known to exist in pure C_{60} at 300 K (refs 5–7, 9, 10) has been ‘frozen out’. The molecules are oriented with twofold axes along cube edges, so the cube faces normal to x , y and z are not equivalent. Twelve Cs atoms per cell are located at (0, 0.5, 0.28) and allowed permutations in space group $Im\bar{3}$. These can be visualized as four-atom ‘cross’ motifs centred on $(\frac{1}{2}, \frac{1}{2}, 0)$ and equivalent positions. This motif also has a twofold axis parallel to the cube edges. A typical motif, as shown in Fig. 2, lies in the {001} plane with atoms displaced ± 0.28 along x and ± 0.22 along y from the face centre. Each C_{60} is thus surrounded by 24 Cs atoms, and each Cs is in a distorted tetrahedral environment of four C_{60} s. The ideal stoichiometry is M_6C_{60} . The shortest distance between C_{60} centres is 9.86 and 10.21 Å for K and Cs respectively. These bracket the value of 10.02 Å in the undoped f.c.c. phase^{5–7}. Nearest-neighbour C–Cs distances lie in the range 3.38–3.70 Å; the sum of van der Waals C and ionic Cs radii is 3.2 Å. All the Cs–Cs nearest-neighbour distances are 4.19 Å, considerably greater than the ionic diameter of 3.34 Å.

Alkali-graphite intercalation compounds are generally less dense than their constituents, because a large interlayer dilation overwhelms the effect of adding close-packed layers of heavier atoms^{11,12}. For example, the densities of graphite, K metal and Cs metal are 2.23, 0.86 and 1.87 g cm^{−3} respectively, whereas the saturated intercalation compounds KC_8 and CsC_8 are respectively 0.88 and 0.79 times as dense as graphite. The converse is true for alkali-doped C_{60} despite comparable M/C ratios—the intermolecular nearest-neighbour distance is affected only weakly by doping, and each molecule still has 8 nearest neighbours in the doped phase, compared to 12 in the undoped phase. The crystallographic density of f.c.c. C_{60} is 1.7 g cm^{−3}, whereas the corresponding values for b.c.c. K_6C_{60} and Cs_6C_{60} are 1.30 and 1.81 times the undoped value. This indicates that the average bonding in the two guest–host systems is significantly different; in particular, there are no nearest-neighbour host layers in stage 1 graphite intercalation compounds, and the host–guest–host sandwich thickness is always less than the sum of van der Waals C and ionic M diameters^{11,12}.

A fundamental question in the study of fullerite structures is the nature of the orientationally ordered phase, because icosahedral building blocks cannot simultaneously satisfy space-filling and three-dimensional Bravais-lattice symmetry requirements. Orientational disorder in C_{60} at 300 K removes the symmetry restriction, and the close-packed lattice is therefore a natural consequence. On the other hand, icosahedral viruses often maintain orientational order at 300 K without close packing, crystallizing for example in b.c.c. or monoclinic structures¹³. Apparently the alkali-metal crosses can stabilize the orientationally ordered b.c.c. structure in doped C_{60} by occupying the otherwise empty volume. The electrostatic field associated with

the ionized metal sublattice is almost certainly the driving force for molecular orientational order in the doped structures. Molecular pendant groups can also lead to orientationally ordered phases without a counter-ion sublattice¹⁴.

The electronic structures of different M_xC_{60} compounds will no doubt reflect the differences in guest-host and guest-guest interatomic distances demonstrated by our X-ray results. For example, direct intermolecular overlap is enhanced relative to C_{60} on K doping but reduced by Cs doping, which could be responsible for the lower conductivity of the latter¹. It is significant that no analogous phenomenon exists in the fully ordered phases of doped polymers¹⁵ or intercalated graphite^{11,12}, there all guest atoms have the same local environment, and the average host dilation scales with the size of the guest. The authors of ref. 1 suggest that the low conductivity obtained with Cs doping is due to "the difficulty in incorporating the larger Cs^+ counter-ion (radius 1.67 Å) in the lattice". Their tacit assumption was the persistence of the f.c.c. sublattice of C_{60} molecules, which may be true under their nonequilibrium growth conditions near 25 °C. Figure 1 clearly shows, however, that K and Cs react quite similarly with solid C_{60} under equilibrium conditions at 200–225 °C.

The intercalation analogy is also inconsistent with the fact that the 300-K conductivity varies strongly with M^+ ; in graphite intercalates, the normal metallic properties are largely independent of $M^{11,12}$. Our results suggest instead that these differences are due to small but different overlaps between C_{60} π orbitals on adjacent molecules. We also note that the proper comparison of electronic conductivities in an ionic-salt model is between K-doped C_{60} (presumed isotropic) and, for example, the c -axis value of KC_8 , of the order of $1,000\text{ S cm}^{-1}$ at 300 K (ref. 16). This is similar to the value reported for K-doped C_{60} samples that are at best crystalline fullerite and perhaps even amorphous; the intrinsic conductivity of a crystalline fullerite intercalation compound is doubtless much higher, perhaps too large to reconcile with intermolecular hopping in a charge-transfer salt. As

KC_8 is an anisotropic type II superconductor, further tests of the analogy may be possible by comparing critical fields¹⁷.

The results presented here suggest that the bonding in alkali-doped C_{60} is not governed simply by van der Waals intermolecular contact, and therefore that the electronic spectra, for the fully doped samples at least, may not be amenable to rigid-band arguments and valence-electron counting. We have yet to determine if the ordered phases are superconducting; it may turn out that disorder is an essential feature of the superconducting state in these unusual guest-host solids.

During the review process, we learned that Holczer *et al.*¹⁸ found up to 40% diamagnetic shielding with respect to bulk superconductivity in doped samples with global composition $K_{3.1}C_{60}$, and no diamagnetism at a global composition K_6C_{60} . Their best samples show at least ten times more shielding than the samples from AT&T Bell Laboratories² of the same nominal composition. This encouraging result underscores the need for complete structural and chemical identification of the superconducting phase (or phases). □

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Prediction of fullerene packing in C_{60} and C_{70} crystals

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RECENT breakthroughs^{1,2} in synthesizing large amounts of C_{60} , C_{70} and other fullerenes³ have made possible studies of the structures and properties of fullerene crystals. Using a force field developed recently for sp^2 carbon atoms, we predict here the crystal structures and cohesive energies for close-packed crystals of C_{60} and C_{70} . We predict, and confirm from calculations, that for C_{60} face-centred cubic (f.c.c.) packing is more stable than hexagonal close-packing (h.c.p.), by $0.90\text{ kcal mol}^{-1}$, whereas for C_{70} h.c.p. is more stable than f.c.c. by $0.35\text{ kcal mol}^{-1}$. The cubic structure of C_{60} undergoes an orthorhombic distortion to space group $Cmca$ at 0 K. At higher temperatures there is rapid reorientation (but not free rotation) of C_{60} molecules, suggesting that above about 200 K a phase transition occurs to an orientationally disordered, f.c.c. structure (with a room-temperature lattice parameter of 14.13 Å). This may correspond to the first-order transition observed⁴ at 249 K. The threefold axes of the C_{60} molecules in the low-temperature structure are not aligned with the threefold crystallographic axes.

To understand the packing of fullerenes it is useful to consider the energetics of fullerene dimers in various orientations. For the C_{60} dimer the most energetically favourable interaction

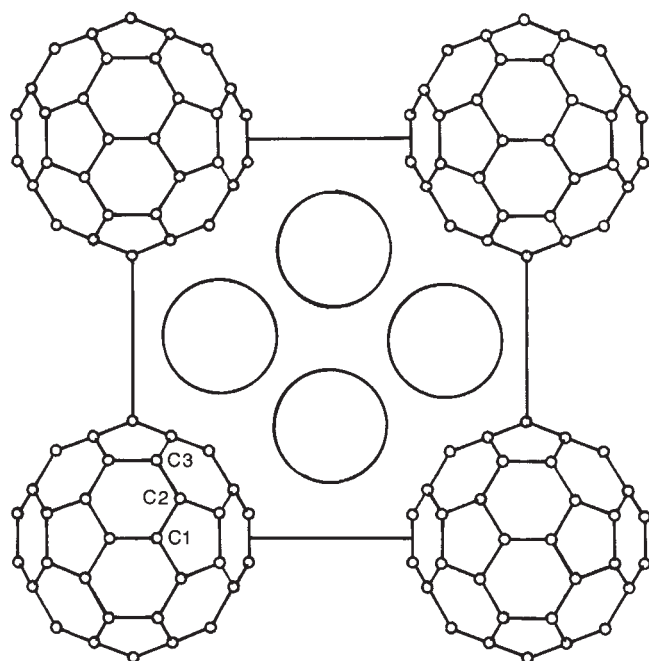


FIG. 2 Schematic of the cubic unit-cell face normal to the z direction, derived from Rietveld refinement of the Cs_xC_{60} profile in Fig. 1. Large circles represent Cs^+ in scale with the cube edge. Small circles are C atoms, not to scale. An equivalent C_{60} molecule is centred at $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ (not shown). Cs (or K) coordinates ((clockwise from top) are $(0.5, 0.72, 0)$, $(0.78, 0.5, 0)$, $(0.5, 0.28, 0)$ and $(0.22, 0.5, 0)$ with respect to an origin at the bottom left corner. Faces normal to the x or y directions may be visualized by rotating the diagram $\pm 90^\circ$. This is a consequence of the molecular orientation of C_{60} , with twofold axes along cube edges.

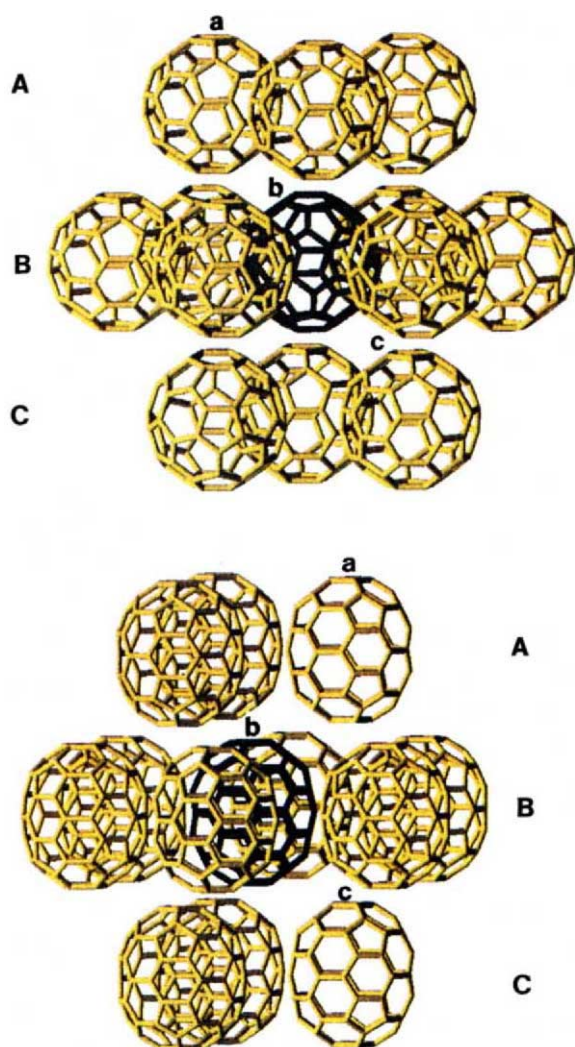


FIG. 1 Top, calculated crystal structure of C_{60} fullerene. Shown (in blue) is one of the four molecules of the f.c.c. unit cell, surrounded by its optimum 12 neighbours. Bottom, calculated crystal structure of C_{70} fullerene. Shown (in blue) is one of the four molecules of the h.c.p. unit cell, surrounded by its optimum 12 neighbours.

occurs for two hexagonal faces in contact (the same orientation, but with the centres shifted by $0.92 \text{ \AA}$ towards one edge). The equilibrium bond distance is $R_e = 9.84 \text{ \AA}$ (centre to centre) and the bond energy is $D_e = 7.30 \text{ kcal mol}^{-1}$. This leads to a separation of the faces of $3.27 \text{ \AA}$ and a closest atom-atom distance of $3.36 \text{ \AA}$. The least favourable orientation leads to an energy of $6.10 \text{ kcal mol}^{-1}$, about 1 kcal mol^{-1} smaller than the best.

For the C_{70} dimer, the optimal interaction is with the long axes parallel. The waist of the monomer involves both concave and convex graphite-like regions, and the best interaction of the dimer ($D_e = 8.97 \text{ kcal mol}^{-1}$, $R_e = 9.94 \text{ \AA}$) has a convex side matched with a concave side. Here the worst orientation leads to an energy of $6.09 \text{ kcal mol}^{-1}$, about 3 kcal mol^{-1} worse than the best.

Because of non-crystallographic symmetries (fivefold axes), it is not possible for these fullerenes to retain the optimal dimer orientation in the closest-packed structures. The symmetry of the fullerene does, however, affect the packing. To understand why C_{60} prefers f.c.c. whereas C_{70} prefers h.c.p., consider a closest-packed layer B and the optimized closest-packed layer A on top as in Fig. 1. The fullerenes orientate so as to optimize interactions, on average, both within and between layers. For C_{60} (symmetry I_h) this leads to hexagonal faces almost in contact (see molecules a and b in Fig. 1a). The inversion symmetry of C_{60} implies that the optimal interaction of the molecule b in layer B with molecules of layer C is to have each c molecule on the opposite side of the optimal b-a interaction. Thus we expect C_{60} to prefer ABCABC (f.c.c.) packing, as in Fig. 1a.

For C_{70} (symmetry D_{5h}), optimal packing in a closest-packed plane is achieved with the long (fivefold) axis perpendicular to the plane (see Fig. 1b), with the horizontal reflection σ_h in the plane. In this case the optimal interaction of molecules b and c is on the same side as the optimal b-a interaction. Thus the best packing is for layers C and A to be the same, leading to ABABAB (h.c.p.) packing for C_{70} , as in Fig. 1b.

There are no definitive crystal structures published for C_{60} or C_{70} . Krätschmer *et al.*¹ concluded that the structure for C_{60} is h.c.p., but their data did not really distinguish between h.c.p. and f.c.c.. Stoddart⁵ quoted unpublished work by F. Diederich, indicating that f.c.c. is more stable for C_{60} . Indeed, Diederich (personal communication) observes only cubic crystal habits for C_{60} and only hexagonal habits for C_{70} , as would be expected from our predictions (diffraction data from the crystals are not of sufficient quality for structural analysis). There are other, as yet unpublished, experimental studies supporting the f.c.c. structure for C_{60} (for example, refs 4, 6). The predicted differences

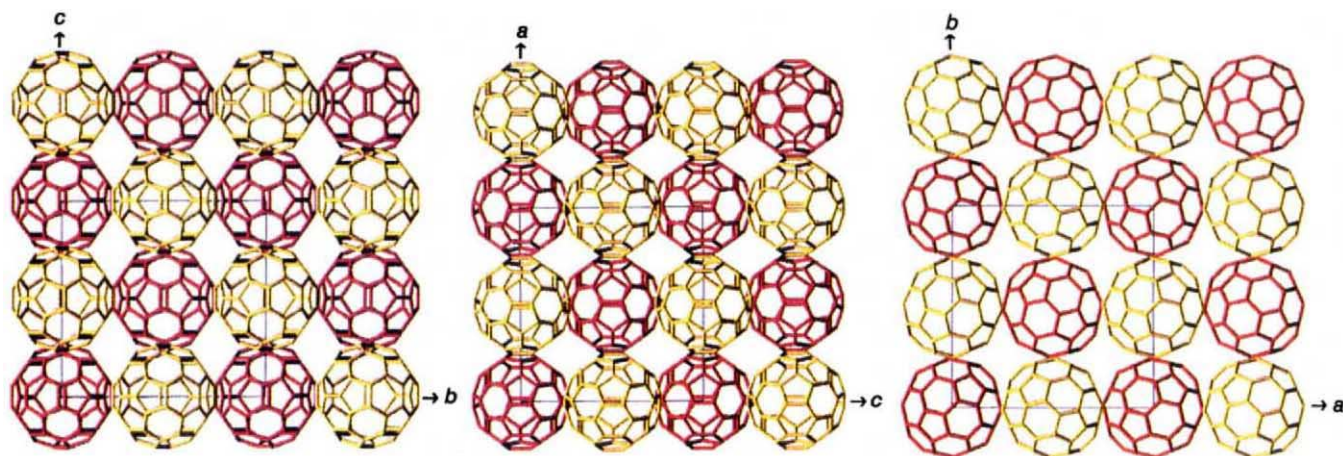


FIG. 2 Optimal f.c.c. structure: the f.c.c. unit cell of C_{60} projected along the three axes: left, [100]; centre, [010]; right, [001]. The orthorhombic unit cell

is shown with blue lines. Balls of different colour are in different planes.

TABLE 1 Properties of fullerene crystals

Structure*	Energy (kcal mol ⁻¹)	Density (g cm ⁻³)	Cell parameters (Å and degree)					
			<i>a</i>	<i>b</i>	<i>c</i>	α	β	γ
C ₆₀	f.c.c.-best	0.000†	14.22§	14.22	13.56	90.01	90.00	90.00
	f.c.c.-2nd	0.312	14.27	14.28	13.50	90.01	90.00	90.01
	f.c.c.-3rd	0.505	14.32	14.26	13.50	90.00	90.00	89.72
	f.c.c.-4th	0.660	14.36	14.26	13.92	89.70	89.99	89.83
	h.c.p.	0.903	14.32	19.77¶	16.31	90.44	90.98	119.39
	f.c.c.-ideal	5.078	10.06	10.06	10.06	60.00	60.00	60.00
	b.c.c.	5.279	9.78	9.78	9.77	109.47	109.47	109.47
C ₇₀	h.c.p.	0.000‡	9.92	20.09¶	18.52	90.01	90.00	119.61
	f.c.c.	0.351	14.84	14.84	14.76	85.44	85.44	84.23
	b.c.c.	10.682	10.13	10.26	10.19	107.41	107.33	107.68

* All calculations allowed four independent molecules per cell (except b.c.c. where there was only one independent molecule per rhombohedral cell).

† Total cohesive energy is 43.883 kcal mol⁻¹ using the minimum energies for gas phase and crystal. Correcting for zero-point energy and the temperature dependence of C_V , S and H , we obtain $\Delta H_{707K} = 40.9$ kcal mol⁻¹.

‡ Total cohesive energy is 49.506 kcal mol⁻¹ using the minimum energies for gas phase and crystal. Correcting for zero-point energy and the temperature dependence of C_V , S and H , we obtain $\Delta H_{739K} = 46.4$ kcal mol⁻¹.

§ Averaging the lattice parameters leads to $a=b=c=14.00$ Å at 0 K and $a=b=c=14.13$ Å at 300 K.

|| b.c.c. is not locally stable, and we restricted the calculation to ideal angles.

¶ This h.c.p. unit cell has four independent molecules per cell.

in energy between h.c.p. and f.c.c. suggests that compression annealing might be used to obtain samples of fullerenes with higher crystallinity. The calculated densities for f.c.c. C₆₀ of 1.745 g cm⁻³ at 0 K and 1.697 g cm⁻³ at 300 K (from molecular dynamics) are in agreement with the observed density of 1.678 g cm⁻³ at room temperature¹.

To interpret and predict various properties for fullerene molecules and crystals, (including vibrational spectroscopy^{1,2,7}, crystal structure analysis¹, NMR⁸ and scanning tunnelling microscopy^{9,10}), we developed a force field¹¹ for sp^2 carbon centres by fitting experimental lattice parameters, elastic constants and phonon frequencies for graphite. This force field uses Lennard-Jones 12-6 van der Waals interactions ($R_v = 3.8050$, $D_v = 0.0692$), Morse bond stretches ($R_b = 1.4114$, $k_b = 720$, $D_b = 133.0$), cosine angle bends ($\theta_a = 120$, $k_{\theta\theta} = 196.13$, $k_{r\theta} = -72.41$, $k_{rr} = 68$) and a twofold torsion ($V_t = 21.28$), where all distances are in Å, all angles in degrees, all energies in kcal mol⁻¹, and all force constants in kcal mol⁻¹, Å and radian units. For more details on the forms of these potentials see ref. 12. All calculations in this paper were carried out using POLYGRAF (Molecular Simulations Inc., Sunnyvale, California) in conjunction with additional vibrational analysis software written by N.K., S. Dasgupta and W.A.G. The calculations were carried out on Silicon Graphics 4D/380 and 4D/25 graphics workstations.

Using this force field and allowing four independent molecules per unit cell (permitting these molecules to reorientate with respect to each other), we calculated the optimal crystal structures for both simple types of closest packing, h.c.p. and f.c.c. We also considered b.c.c. packing (8 neighbours instead of 12), but this led to much higher energies. In these calculations we used periodic boundary conditions with accuracy-bounded convergence acceleration¹³ for the long-range van der Waals interactions. All $4 \times 60 \times 3 = 720$ internal degrees of freedom, plus the six cell degrees of freedom, were optimized simultaneously. We calculated all second derivatives of the energy with respect to these 726 degrees of freedom to determine whether the structures were locally stable (that is, all second derivatives positive).

The optimum structure for C₆₀ is shown in Fig. 1a and Fig. 2, where the blue lines indicate the unit cell. This structure is an orthorhombic distortion (space group $Cmca$) of f.c.c.. Although we allowed four C₆₀ molecules per unit cell to be independent, we find that the optimal structure has two equivalent pairs of molecules, as indicated in Figs 1a and 2. We found three other locally stable f.c.c. structures (all second derivatives positive) at energies (per C₆₀) 0.31, 0.51 and

0.66 kcal mol⁻¹ higher. The h.c.p. structure (also locally stable) was 0.90 kcal mol⁻¹ higher in energy.

From symmetry, one might expect f.c.c. C₆₀ to orientate each molecule with the diagonal (threefold) axes parallel to the threefold axes of a cubic crystal (in this orientation, the twofold axes of C₆₀ are along the cubic axes). Our calculations show, however, that this structure is not stable (leading to three negative eigenvalues of second-derivative matrix (hessian), corresponding to rotations of the C₆₀s). We will refer to this as the 'ideal' f.c.c. structure. As shown in Table 1, the energy of the ideal f.c.c. structure is fairly high (5.08 kcal mol⁻¹). This is close to that for b.c.c. and five times as high as for h.c.p. The reason is that this packing does not allow contact between the hexagonal faces; instead, it leads to poor nearest-neighbour interactions.

In the optimal f.c.c. structure, each C₆₀ leads to eight good interactions (with hexagonal rings in contact as in the optimal dimer) and four contacts that are energetically worse by ~ 1 kcal mol⁻¹. The average energy and distance for the good interactions are 7.11 kcal mol⁻¹ and 9.83 Å (compared with 7.20 and 9.84 for the dimer), whereas the average values for the four poor interactions are 6.12 kcal mol⁻¹ and 10.06 Å. On the other hand, the ideal f.c.c. structure leads to 12 interactions that are nearly the worst possible (6.01 kcal mol⁻¹ and 10.06 Å). This difference of $8 \times 1.1 \times \frac{1}{2}$ accounts for 4.4 kcal mol⁻¹ of the 5.1-kcal mol⁻¹ difference in energy. (Interestingly, for K₃C₆₀ we find¹⁴ a f.c.c. unit cell with exactly this ideal structure of the C₆₀ units; here the electrostatic and van der Waals interactions between K and C₆₀ dominate.)

The optimal f.c.c. structure leads to a distorted orthorhombic crystal structure at 0 K, and the other low-lying structures with f.c.c. packing (at 0.3–0.7 kcal mol⁻¹) also distort at low temperature. Molecular dynamics calculations carried out for short periods (20 ps) show that at low temperature (100–200 K), the C₆₀ molecules librate locally around the equilibrium and do not rotate. But near room temperature (300–400 K), the C₆₀ molecules tunnel from one favoured orientation to another. Such orientational fluctuations lead to an average structure that is cubic (lattice parameter $a = 14.13$ Å at 300 K) with orientational disorder (but not free rotation) among the C₆₀ molecules. (Note that this cubic structure is not the ideal f.c.c. structure.) Thus we expect a phase transition to occur between the distorted orthorhombic form (at low temperature) and the cubic, orientationally disordered form (at room temperature). The first-order transition observed⁴ at 249 K might correspond to this transition. The transition temperature should increase and the lattice parameter decrease as better-quality crystals are produced.

For C_{70} the interactions in the crystal are much more anisotropic than for C_{60} . The interactions within the plane have $R_e = 9.92\text{--}10.06\text{ \AA}$ (10.00 average) and $D_e = 8.23\text{--}8.79\text{ kcal mol}^{-1}$ (8.47 average), whereas those out-of-plane have $R_e = 10.83\text{--}10.96\text{ \AA}$ (10.91 average) and $D_e = 6.75\text{--}7.18\text{ kcal mol}^{-1}$ (6.90 average). This can be compared with the optimal dimer interactions of $R_e = 9.94\text{ \AA}$ and $D_e = 8.97\text{ kcal mol}^{-1}$.

After submission of this manuscript, ref. 15 reported heats of sublimation of $\Delta H_{707K} = 40.1 \pm 1.3\text{ kcal mol}^{-1}$ for C_{60} and $\Delta H_{739K} = 43.0 \pm 2.2\text{ kcal mol}^{-1}$ for C_{70} . Our calculations lead to a cohesive energy of $43.9\text{ kcal mol}^{-1}$ for C_{60} and $49.5\text{ kcal mol}^{-1}$ for C_{70} . Correcting for zero-point energy and temperature dependence of the specific heat capacity C_v , entropy S and H for both the crystal and free molecule, we calculate $\Delta H_{707K} = 40.9\text{ kcal mol}^{-1}$ for C_{60} and $\Delta H_{739K} = 46.4\text{ kcal mol}^{-1}$ for C_{70} , in excellent agreement with experiment. $\square$

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Diffraction-like effects in NMR diffusion studies of fluids in porous solids

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THE transport of fluids in porous media is of importance in a wide range of areas, such as oil recovery, heterogeneous catalysis and biological perfusion. The pulsed gradient spin-echo (PGSE) NMR technique has been used for many years to characterize diffusion and flow in such systems^{1–3}. The analogy between NMR measurements in a field gradient and diffraction has been pointed out in the context of NMR imaging⁴ and, more recently, diffraction-like effects in the PGSE experiment have been discussed for diffusion in both impermeable⁵ and connected⁶ structures. The gradient pulse area plays the role of a wavevector, q , which can probe the structure in which the fluid diffuses. Here we report experimental confirmation of these predicted effects from proton NMR studies of a water-saturated, orientationally disordered, loosely packed array of monodisperse polystyrene spheres. The PGSE-NMR experiments may thus be used to provide an indirect, averaged

image of the internal structure of porous solids at a resolution higher than that achievable with conventional NMR imaging. This is particularly advantageous for measurements on large samples, as the resolution available with the PGSE method depends only on the available range of gradient pulse amplitude and duration and is unconstrained by the factors determining resolution in conventional NMR imaging.

The PGSE-NMR experiment is illustrated in Fig. 1a. It comprises two radiofrequency (r.f.) pulses and two magnetic field gradient pulses of amplitude g and duration δ , separated by Δ . The r.f. pulses induce and maintain phase coherence in the precessing nuclear spins. The first field gradient pulse gives phase shifts to the spins proportional to the initial positions of the molecules in the direction of the gradient. The second field gradient pulse, together with the 180° r.f. pulse, again shifts the phase of each spin by an amount proportional to its final position but in the opposite sense. Spins on atoms that remain static in the time between the two field gradient pulses therefore suffer no net phase shift. The statistics of the displacements of those that have moved determine their net phase shift and hence their contribution to the signal. The echo amplitude, $E_\Delta(q)$, is usually written as

$$E_\Delta(q) = \int \int \rho(\mathbf{r}) P_s(\mathbf{r}; \mathbf{r}', \Delta) \exp(i2\pi\mathbf{q} \cdot (\mathbf{r}' - \mathbf{r})) d\mathbf{r} d\mathbf{r}' \quad (1)$$

where $\mathbf{q} = (2\pi)^{-1}\gamma g\delta$, γ being the nuclear magnetogyric ratio. P_s is the conditional probability density that a spin originating at $\mathbf{r}$ at the time of the first gradient pulse will migrate to $\mathbf{r}'$ by the time of the second, thus acquiring a residual phase shift $2\pi\mathbf{q} \cdot (\mathbf{r}' - \mathbf{r})$. $\rho(\mathbf{r})$ is the initial spin density function. There is therefore a Fourier relationship between the spectrum of spin displacements and $E_\Delta(q)$ (ref. 7). In the experiments described here, a single gradient direction was used so that it is appropriate to describe the motion in terms of displacements along the z -axis parallel to $\mathbf{q}$.

To appreciate how the analogy with diffraction arises, first consider the case of liquid molecules completely trapped in a pore for which the liquid spin density function is $\rho(\mathbf{r})$. Provided that the time Δ is sufficiently long that the migrating molecules experience all possible locations in the pore, then P_s is independent of starting position and reduces to $\rho(\mathbf{r}')$. As $\Delta \rightarrow \infty$, Equation (1) yields

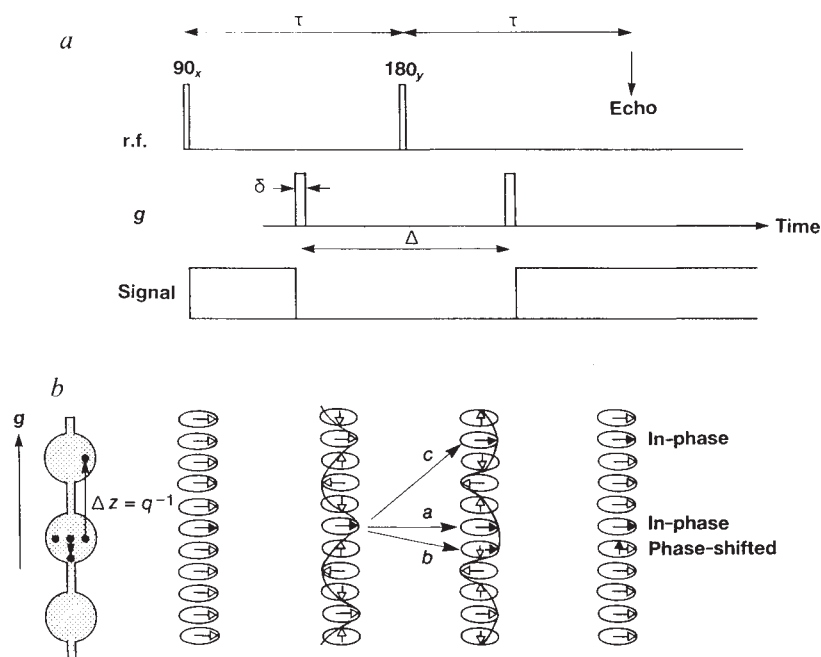
$$E_\infty(q) = |S_0(q)|^2 \quad (2)$$

where $S_0(q)$ is the q -space Fourier transform of $\rho(\mathbf{r})$ (refs 5, 6). Equation (2) states that the PGSE experiment for restricted motion in an enclosed pore is mathematically analogous to optical diffraction by a single slit⁵. In passing, we note that in conventional NMR imaging, where the field gradient G is applied for a time t , the signal is $S(\mathbf{k})$ where $\mathbf{k} = (2\pi)^{-1}\gamma Gt$ (ref. 4). This direct measurement of $S(\mathbf{k})$ must be contrasted with the PGSE-NMR experiment in the limit of long diffusion time as it is clear from equation (2) that in the latter case the phase information is lost. This loss is compensated, however, by the possibility of achieving higher resolution.

Suppose now that the pores are connected so that a spin-bearing molecule originating in one pore can migrate to other pores. Diffusion between pores effectively changes the single-slit 'diffraction' into a multi-slit pattern where the elements of the 'diffraction grating' are weighted according to a diffusion envelope, $C(Z, \Delta)$, which describes the probability that a molecule will migrate to a pore displaced by a distance Z from the starting pore after a time Δ (ref. 6). This dynamic displacement must be distinguished from the positions of the molecules along the gradient direction, which we denote by z . Provided that the time scale for diffusion within a pore is much shorter than that for diffusion between pores, P_s becomes a product of the pore density and the probability of jumps between pores. This is the 'pore-equilibration' assumption, which will be valid

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FIG. 1 *a*, Sequence of events in the PGSE-NMR experiment (explained in the text). Note that in reality the signal following the first r.f. pulse will decay because of inhomogeneities in the static magnetic field and that, similarly, the signal following the second field gradient pulse will have the form of a spin echo, rising to a maximum at $t = 2\tau$. *b*, Progression of phases of spins at different positions along the gradient direction. The white arrows correspond to spins that have not changed positions over the time Δ . Such spins have their phase shifts perfectly refocused. The black arrows correspond to three possible displacements of a spin starting from the central pore: *a*, stationary, *b*, arbitrary displacement and *c*, a displacement corresponding to q^{-1} , the gradient wavelength. Displacement *c* gives rise to the same perfect refocusing as for stationary spins. A sample that favours displacement corresponding to q^{-1} (such as the periodic porous object shown on the left) will exhibit a coherence peak in the echo amplitude when q^{-1} equals one pore spacing.



provided that $a^2/D_0 \ll b^2/D_{\text{eff}}$ where a and b are the pore size and pore spacing, and D_0 and D_{eff} describe the self-diffusion coefficients for brownian motion within the pore and for long-range migration between the pores, respectively. Thus

$$E_{\Delta}(q) = |S_0(q)|^2 [F\{L(Z)\} \otimes F\{C(Z, \Delta)\}] \quad (3)$$

where $\otimes$ represents convolution, F denotes Fourier transformation and $L(Z)$ is the 'lattice correlation function' describing the relative positions of pore centres.

The correspondence with X-ray diffraction is as follows. $|S_0(q)|^2$ plays the part of an average atomic form factor, in this case arising from 'diffraction' caused by the local pore density $\rho_0(z)$. $F\{L(Z)\}$ is the reciprocal lattice which is broadened by

$F\{C(Z, \Delta)\}$. This broadening arises because the number of 'scattering centres' in the lattice increases with time as the molecules diffuse to more distant pores. In a disordered structure, for example, where the pore spacing b is irregular, the sharpest coherence peak (maximum in $E_{\Delta}(q)$ as a function of q) will be at $q \equiv |q| = b^{-1}$, so that the condition $\Delta \approx b^2/2D_{\text{eff}}$ corresponds to the optimum evolution time for observing such structure. It should be noted that D_{eff} can be measured by observing $E_{\Delta}(q)$ in the low- q limit, thus probing the long-range molecular displacements. By contrast, $|S_0(q)|^2$ will be apparent as a modulation in the high- q regime.

We have performed a series of PGSE-NMR measurements on the diffusion of water in an assembly of monodisperse polystyrene spheres (7516A; Duke Scientific). These spheres had been packed by gentle centrifugation giving a porosity of 44%, which is consistent with random loose packing³. Therefore, the pore spacing b is roughly equal to the sphere diameter, 15.8 μm . The porous solid so produced has long-range orientational disorder so that $L(Z)$ is characteristic of a pore 'glass' with short-range (essentially first-shell) correlations. In addition, the pores have rather unusual shapes with a size of $\sim \frac{1}{3}$ the sphere diameter⁹.

Despite such random orientation, a first-order coherence peak can be expected by analogy with X-ray diffraction from a glass.

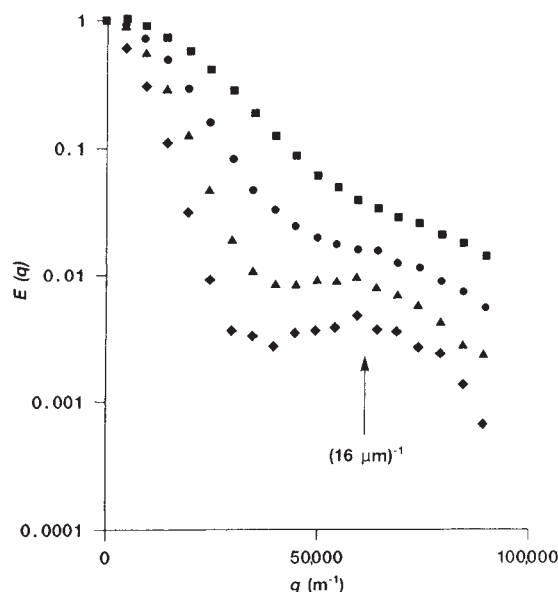


FIG. 2 Echo intensities $E_{\Delta}(q)$ as a function of the gradient wavevector q for a series of diffusion times Δ . The low- q data yield a value for D_{eff} of $2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$. The feature at $q = 60,000 \text{ m}^{-1}$ corresponds to a pore separation distance of 16 μm , which is consistent with random loose packing. ■, 20 ms; ●, 40 ms; ▲, 70 ms; ◆, 110 ms.

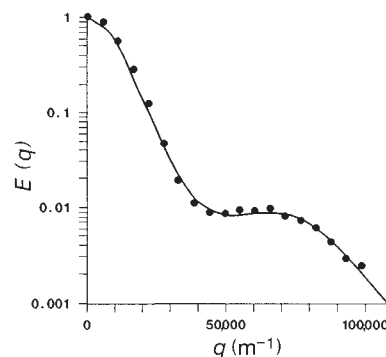


FIG. 3 Echo intensities $E_{\Delta}(q)$ at $\Delta = 70 \text{ ms}$. The data have been fitted to a theoretical curve obtained using equation (4) in a nonlinear least squares fit.

Figure 2 shows $E_{\Delta}(q)$ for a succession of times Δ from 20 to 110 ms. From the low- q data, we find $D_{\text{eff}} = 2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$, so the timescale ranges from $0.3(b^2/2D_{\text{eff}})$ to $1.8(b^2/2D_{\text{eff}})$. A coherence peak at $q \approx (16 \mu\text{m})^{-1}$ is clearly visible at values of Δ sufficiently large (≥ 70 ms) for the molecules to diffuse from the starting pore to a first neighbour.

A quantitative fit to the data is possible using equation (3). A simple approximation to $C(Z, \Delta)$ is to assume a gaussian envelope⁶, but a more precise description can be made if we consider the cumulative effects or successive pore hops. This pore-hopping formalism yields an approximate closed-form expression for $E_{\Delta}(q)$ in the case of the pore glass, namely

$$E_{\Delta}(q) = |S_0(q)|^2 \times \exp \left[-\frac{6D_{\text{eff}}\Delta}{b^2 + 3\xi^2} \left(1 - \exp(-2\pi^2 q^2 \xi^2) \frac{\sin(2\pi qb)}{2\pi qb} \right) \right] \quad (4)$$

where b is the mean and ξ the standard deviation of the distance from the starting pore to the nearest-neighbour pore shell. A more detailed description of pore-hopping theory will be published elsewhere. Figure 3 shows the data for $\Delta = 70$ ms, together with a theoretical curve obtained by using equation (4) in a nonlinear least-squares fit. For simplicity, the form factor chosen corresponds to a spherical pore of diameter a and is thus rather unrealistic. Nonetheless, the fit is fairly good and yields structural parameters $b = 16.1 \mu\text{m}$, $a = 5.6 \mu\text{m}$ and $\xi = 2.85 \mu\text{m}$, which correspond to the physical dimensions of the packed sphere array.

We have performed an inverse Fourier transformation on the $\Delta = 110$ -ms data of Fig. 2, using three-dimensional projection reconstruction and assuming the system to be isotropic. This gives an 'image' of the spherically averaged autocorrelation function for the pores convoluted with the diffusion-weighted three-dimensional lattice correlation function (as opposed to the projection, $L(Z)$, of this function along a single axis). Figure 4 shows a slice through the centre of this three-dimensional 'image'.

For the q -space imaging of dynamic displacements, the entire sample contributes to the 'image' signal-to-noise ratio; the resolution

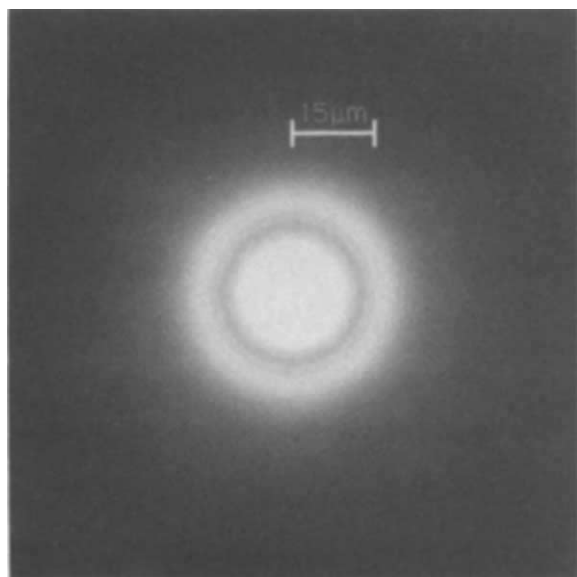


FIG. 4 'Image' of the pore autocorrelation function convoluted with the diffusion-weighted three-dimensional lattice correlation function. The 'image' was obtained by Fourier transformation of the 110-ms data of Fig. 2. The bright inner circle and the first bright ring correspond to the starting pores and the first-neighbour pore shells of the 16- μm spheres respectively.

is limited only by the available magnitude of q . This is in contrast to NMR imaging in k -space, where increasing the resolution by increasing the imaging gradient results in a deteriorating signal-to-noise ratio in each volume element of the 'image'. We envisage that q -space imaging could realistically achieve sub-micrometre resolution.

The observation of a coherence peak as the gradient is increased in a PGSE experiment is remarkable and completely contrary to the usual expectation of monotonically decreasing $E_{\Delta}(q)$ with increasing q . Such a peak arises because of structural features in the porous solid. A significant number of molecules moving one pore spacing suffer no net phase shift when $qb \approx 1$. We are unaware of any previous measurement in which $E_{\Delta}(q)$ increases with increasing q . □

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Air entrainment and dissipation in breaking waves

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WAVE breaking transfers momentum from the atmosphere (winds) to the ocean (currents)^{1,2} and entrains air in bubbles which are believed to generate and scatter underwater sound^{3–5}. Wave breaking and the associated entrainment of air in bubbles are also thought to be important in heat and gas transfer across the air–sea interface^{6–8}, but the lack of detailed measurements of air entrainment in bubbles has impeded our understanding of the effect of wave breaking on these processes. Here we present measurements of air entrainment by controlled deep-water breaking waves, which show that the bubble plumes generated by breaking waves contain volume fractions of air that are many orders of magnitude greater than expected. Bubble plumes with such large void fractions may be the source of low-frequency sound in the ocean⁹. We conclude that the processes of surface-wave evolution and air entrainment are dynamically coupled, and that the contribution of bubbles to air–sea gas transfer and to sound propagation may be seriously underestimated if the existence of these plumes of large bubbles is not taken into account.

Experiments were conducted in a wave channel 25-m long and 0.7-m wide filled with fresh water to a depth of 0.6 m. The experiment consisted of generating breaking waves and mapping the bubble plume created by breaking for three different amplitudes of the same wave packet (see Table 1 for wave-packet characteristics). The breaking waves were produced by a piston-type wavemaker. Details of wave generation and photographs of breaking waves are given by Rapp and Melville². The technique gives very repeatable wave profiles and energy dissipation, and we found it to give fairly repeatable bubble plumes. The volume fraction of air (void fraction, given in % hereafter) in an air–water mixture can be measured by making use of the large difference in electrical conductivity between air and water. This technique has been used in closed-conduit two-phase flows,

and reviews can be found in the literature^{10,11}. We used essentially the same technique to map the void-fraction distribution in bubble plumes generated by breaking waves. Typical wave-gauge and void-fraction time series are shown in Fig. 1. Additional details of the probe and the experimental procedures will be published elsewhere.

Contour maps showing the evolution of the void-fraction distribution can be assembled from the individual time series (Fig. 2). Measurements over the full range 0–100% of void fractions have been observed, and void fractions of >20% for all three wave amplitudes were sustained for up to half a wave period after breaking. These measurements show that the volume fractions of air near the surface within one wave period following breaking are many orders of magnitude greater than the time-averaged (over ~10 min) values measured in the field¹². It is well known that the speed of sound in water can be reduced by an order of magnitude in the presence of void fractions as low as 1%. Our results therefore suggest caution in using time-averaged void-fraction measurements to infer sound propagation through the surface bubble layer (which acts as an acoustic waveguide¹³).

The important parameters describing the bubble plume are presented in Fig. 3. The total volume of entrained air, V , shown in Fig. 3a is computed from

$$V = b \int_A \alpha \, dA \quad (1)$$

where α is the local void fraction, b is the channel width and A is the cross-sectional area of the bubble plume. The volume of air enclosed in the initial air pocket is conserved for up to a quarter of a wave period after breaking. Degassing of the plume is very rapid, with only 5% of the initially entrained air remaining after a full wave period. The fast rising speed of large bubbles (radius >1 mm) must account for most of the air lost in the first wave period. Laboratory wind-flume experiments¹⁴ have shown the contribution to total flux of carbon dioxide by bubbles to be almost independent of radius in the range 0.02–2 mm despite the fact that larger bubbles are less numerous and have a shorter underwater residence time. The very fast degassing rate of the bubble plume revealed by our experiments indicates that modelling of the contribution of bubbles to total air-sea gas transfer may be considerably underestimated if the transient large bubble population (radius ~1–10 mm) is not considered.

The cross-sectional area of the bubble plume is shown in Fig. 3b. The void-fraction gauge used in our experiments is insensitive

TABLE 1 Characteristics of the three wave amplitudes studied.

ak	t_b (s)	x_b (m)	E_w/b (J m ⁻¹)	E_d/b (J m ⁻¹)	V_0 (cm ³)	$E_d/\rho g V_0 \lambda$	Δx (cm)	Δz (cm)
0.54	14.4	8.05	110.0	17.8	6,860	0.10	5	5
0.45	14.3	7.70	78.0	8.6	3,220	0.10	5	5
0.38	14.3	7.70	58.7	4.3	1,750	0.09	5	5

The slope of the packet² is given by ak . The centre frequency of the wave packet and the bandwidth were both 0.88 Hz. The time from initial motion of the piston is t_b , and x_b is the breaking location downstream of the piston. Both t_b and x_b are defined by the time at which the forward-moving jet strikes the free surface and an air pocket is formed. The volume of the air pocket at $t = t_b^*$ is V_0 (measured from video images at t_b ; because of lack of video resolution for $ak = 0.38$, V_0 was taken as the maximum V measured). E_w is the total pre-breaking wave energy, E_d the total energy dissipated by breaking². $E_d/\rho g V_0 \lambda$ is the ratio of wave energy dissipated to the maximum volume of air entrained normalized by the specific weight of water ρg and the characteristic wavelength λ . The horizontal and vertical resolutions of the void-fraction measurements are Δx and Δz . The channel width, water density and gravity are b , ρ and g .

to void fractions lower than 0.3%. We expect the initial rise in bubble-plume area to be essentially independent of the void-fraction detection threshold, giving an area expansion rate of $28 V_0/bT$ (where T is the characteristic wave period) for the time between 0.1 to 0.3 T . We expect that the rate of decrease of A would be lower in the later stages (after the peak value) if a lower threshold were attainable with the instrument.

The mean void fraction is given by $\bar{\alpha} = V/bA$ (Fig. 3c). Mean void fractions in the bubble plume remain above 1% within the first wave period after breaking. Important changes in the speed of sound are associated with these localized distributions of large void fractions. Such discontinuities have been suggested previously as a possible source of low-frequency sound (<1 kHz) in the ocean¹⁵. Our results indicate that these large discontinuities exist in the laboratory and are likely to be found in the field.

The work required to keep the air entrained against the buoyancy force is

$$E_b = \rho g b \int_A \alpha z \, dA \quad (2)$$

where ρ is the water density, g is the acceleration due to gravity and z is the vertical distance to the free surface. Normalizing

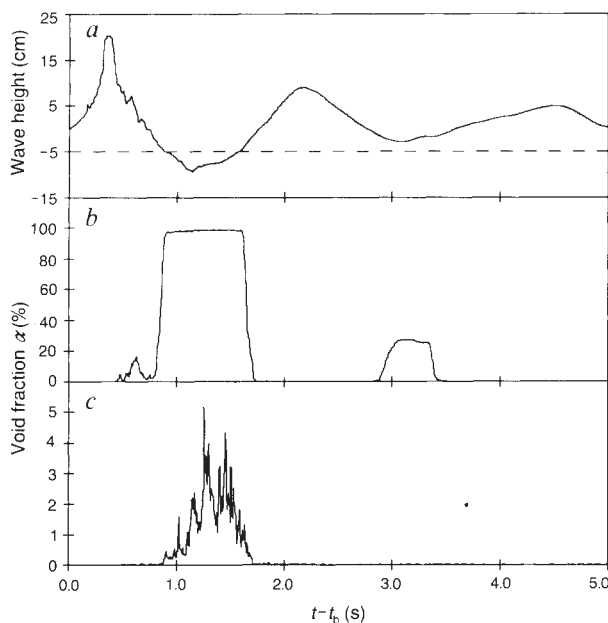


FIG. 1 Typical time series at 200 Hz of a, wave-gauge measurements; b, c, void-fraction measurements at depths -5 cm and -15 cm, respectively, from the still-water level. All data are for the most energetic breaker studied at 0.55 m downstream of x_b . Note how the probe crosses the water surface at 0.8 s in b. 'Surface effect' is shown in b at 3 s. The second wave trough has its water surface at ~-2.5 cm and therefore intrudes into the measuring volume of the void-fraction gauge which is located at -5 ± 3 cm. The effective measuring volume of the probe was a horizontal cylinder ~20 cm long and ~6 cm in diameter.

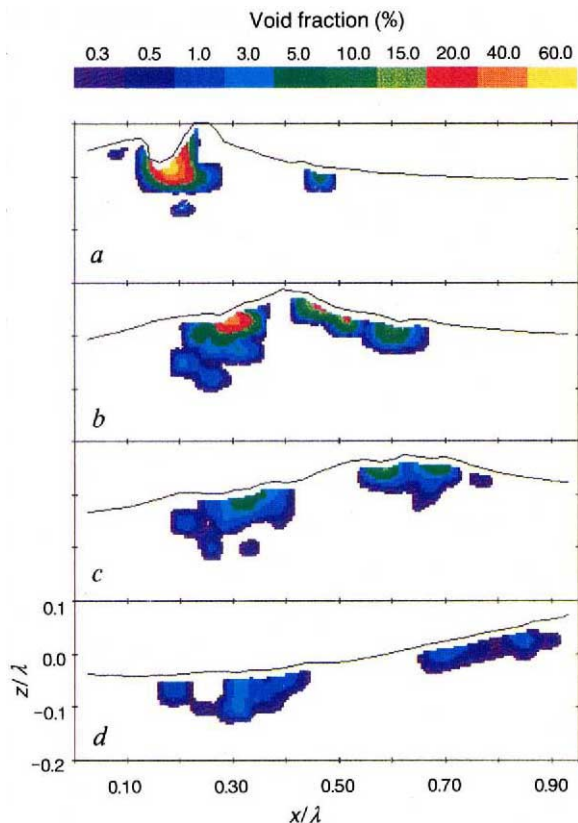
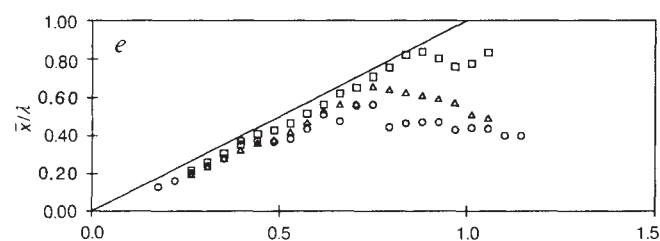


FIG. 3 *a*, Air volume V normalized by V_0 . The data for the three waves collapse onto a simple decaying exponential, $V/V_0 = 2.6 \exp(-3.9(t-t_b)/T)$. *b*, Cross-sectional area A of bubble plume normalized by V_0/b . The data were fitted by using the functional form for V/V_0 divided by that for $b\bar{\alpha}$. (This procedure was used because extrapolation of the curves for V and $\bar{\alpha}$ is evidently better conditioned than extrapolation of the data for A directly.) The fitted curve has equation $Ab/V_0 = 325((t-t_b)/T)^{2.3} \exp(-3.9(t-t_b)/T)$. *c*, Mean void-fraction $\bar{\alpha}$. The data are fitted well by a power law, $\bar{\alpha}(\%) = 0.8((t-t_b)/T)^{-2/3}$ with $\alpha = 100\%$ near $t=0$, as one would expect for the initial air pocket. *d*, Potential energy E_b of the bubble plume, normalized by E_d . Data are fitted with a decaying exponential, $E_b/E_d = 0.7 \exp(-3.6(t-t_b)/T)$. *e, f*, Void-fraction centroids in bubble plume normalized by the wavelength. *e*, Horizontal centroid $\bar{x}$. Solid line is phase speed of the wave, $c = \lambda/T$. *f*, Vertical centroid $\bar{z}$. Note that the vertical centroid does not increase monotonically with ak . Time in all six plots is normalized by the wave period T . Symbols for the three waves: $\circ$, $ak=0.54$; $\square$, $ak=0.45$; $\triangle$, $ak=0.38$.



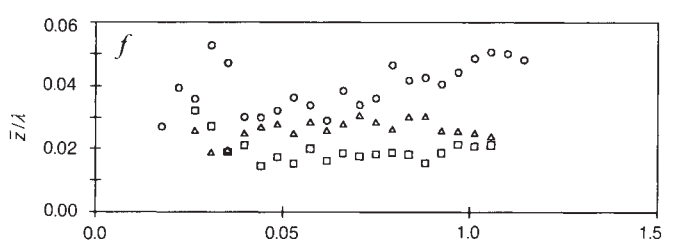
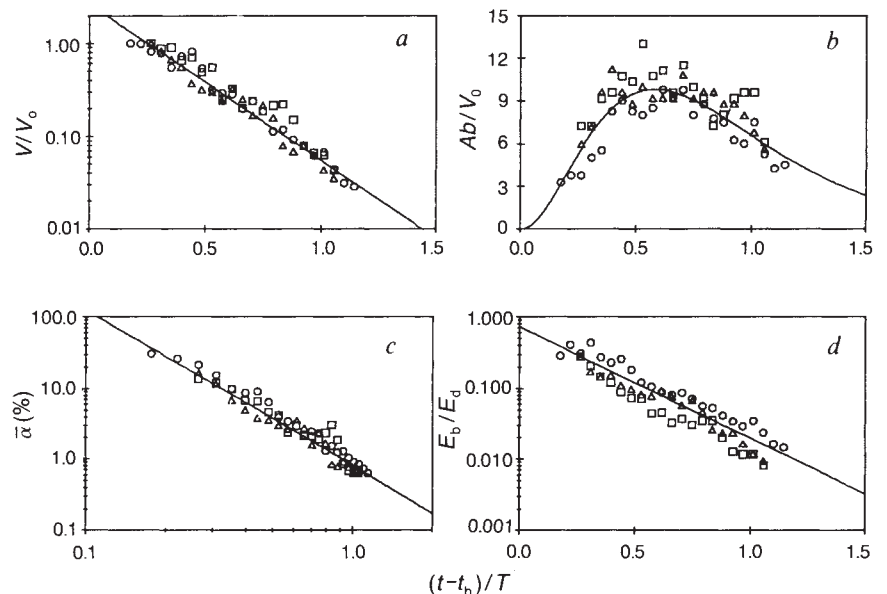
$(t-t_b)/T$

the data by the total wave energy dissipated by breaking, E_d , gives a simple exponential (Fig. 3d). Up to 40% of the total prebreaking wave energy can be lost through breaking¹; our data now show that a large fraction (30–50% and maybe more) of the energy lost is expended in entraining the bubble plume. This is direct evidence that the processes of surface wave evolution and air entrainment have considerable dynamical coupling. The near-constant ratio $E_d/\rho g V_0 \lambda$ for these experiments, as shown in Table 1, implies that the initial volume of air entrained correlates with the energy dissipated. Recent studies^{16,17} have shown that the wave energy dissipated correlates with the acoustic energy radiated and the backscattering of microwave by radar breaking waves. Thus our measurements suggest that it may be possible to quantify air entrainment and gas transfer in the field using acoustic and microwave techniques.

The horizontal and vertical centroids of the void-fraction distribution in the bubble plume $\bar{x}$ and $\bar{z}$, respectively, are

$$(\bar{x}, \bar{z}) = \frac{\int_A \alpha(x, z) dA}{\int_A \alpha dA} \quad (3)$$

where x is the horizontal distance from x_b and z is the depth from the free surface. The horizontal centroid in Fig. 3e moves at roughly the phase speed of the wave for a period $T/2$. In field studies⁵ using sonar, it was found that the cloud in the



early stage after breaking moved at the wind-wave phase speed. The above results are consistent with these findings. The vertical centroid for each packet shown in Fig. 3f is roughly constant, but values for different packets range between 0.015λ and 0.05λ where λ is a characteristic wavelength. This suggests that, at least in the first wave period, the downward advection of fluid may balance the upward motion of the bubbles themselves.

We have shown that the bulk properties of the bubble plume generated by breaking waves scale with the prebreaking wave variables and evolve according to simple functions of time. The close dynamical coupling between wave breaking and air entrainment suggests that models of air-sea gas transfer could be improved by better characterization of breaking surface waves, perhaps through remote sensing methods¹⁷. □

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Constraints from noble-gas contents on the origin of carbonado diamonds

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CARBONADOS are porous aggregates of micrometre-size diamond crystals^{1,2}. Although they are not rare, they have not been found in kimberlites³, and they contain inclusion minerals typical of the Earth's crust, rather than the upper-mantle assemblages commonly found in kimberlite diamonds. Carbonados also have lower $^{13}\text{C}/^{12}\text{C}$ ratios than kimberlite diamonds^{1,2,4}. Although these observations strongly suggest that carbonados were formed in the crust, crustal conditions are generally unlikely to provide pressures and temperatures in the diamond stability field (but see ref. 5 for a possible exception). Other suggestions for the origin of carbonado include the transformation of subducted carbon in the mantle⁶, impact metamorphism of crustal rocks containing organic carbon⁷, and (for very fine-grained carbonado) the irradiation of organic matter by decaying uranium and thorium in uranium-rich phases^{8–10}. Here we present further evidence for a crustal connection, in the form of noble-gas data for four carbonados from Brazil and Africa.

We find that all four contain large amounts of implanted xenon and krypton from ^{238}U fission, nucleogenic neon and ^4He , and tightly trapped atmospheric noble gases, from which we conclude that these carbonados formed in a uranium-rich crustal environment.

We studied two carbonados from Brazil and two from Africa, all from different localities. Before the noble gas experiments, samples were thoroughly cleaned with hot HNO_3 and HF. Noble gases were extracted by heating the samples at temperatures up to 2,000 °C (graphitization temperature is 1,900 °C) and analysed by mass spectrometry. Table 1 gives the analytical data and details of the method. The isotopic compositions of neon, krypton and xenon are distinctly different from those in the atmosphere. The extraordinary neon isotope ratios can be attributed to nuclear reactions (Wetherill reactions)¹¹ such as $^{18}\text{O}(\alpha, n)^{21}\text{Ne}$ and $^{19}\text{F}(\alpha, n)^{22}\text{Ne}$. Assuming that ^{130}Xe , ^{80}Kr and ^{20}Ne are entirely atmospheric, we calculated the excess of the isotopes ^{136}Xe , ^{86}Kr and ^{21}Ne in the samples relative to the values expected if the ratios were atmospheric. We show these in Table 2 together with their uranium and ^4He contents, and $\delta^{13}\text{C}$ values. The excess Xe and Kr isotopes must arise from fission of ^{238}U , and are given the subscript 'fission' in the table. In Fig. 1, we plot $^{136}\text{Xe}/^{132}\text{Xe}$ against $^{134}\text{Xe}/^{132}\text{Xe}$ obtained when the African sample 90706P was degassed by step-heating: the data show that Xe in the diamond is a mixture of atmospheric Xe and Xe arising from ^{238}U fission. Published values¹² of the production ratios for $^4\text{He}/^{136}\text{Xe}$ and $^{136}\text{Xe}/^{86}\text{Kr}$ in spontaneous fission of ^{238}U are 0.3×10^9 and 6.0 respectively. These are well within the range for the observed ratios of $0.27\text{--}0.8 \times 10^9$ and 3.8–12.6. Table 2 also shows the amounts of nucleogenic ^{136}Xe ,

FIG. 1 Step-heating data for Xe. The ratio $^{136}\text{Xe}/^{132}\text{Xe}$ is plotted against $^{134}\text{Xe}/^{132}\text{Xe}$ to form a three-isotope plot. The numbers indicate step-heating degassing temperatures (in 100 °C). Sample, Africa 90706P. This sample was prepared by pulverizing a diamond from the same batch as sample 90706 to fine powder ($<10\ \mu\text{m}$), and thoroughly cleaned with hot HF and HNO_3 to remove possible contaminants. Note that all data lie on a mixing line between atmospheric Xe and ^{238}U fission Xe.

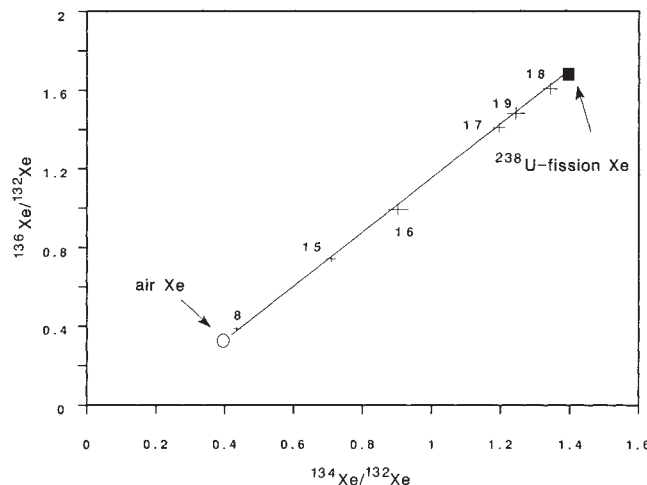


TABLE 1a Isotopic composition of noble gases helium, neon and argon in carbonados

SAMPLE mass (g)	Temperature (°C)	^4He ($10^{-3} \text{ cm}^3 \text{ g}^{-1}$)	^{22}Ne ($10^{-12} \text{ cm}^3 \text{ g}^{-1}$)	$^{20}\text{Ne}/^{22}\text{Ne}$	$^{21}\text{Ne}/^{22}\text{Ne}$	^{36}Ar ($10^{-9} \text{ cm}^3 \text{ g}^{-1}$)	$^{38}\text{Ar}/^{36}\text{Ar}$	$^{40}\text{Ar}/^{36}\text{Ar}$
Brazil, Poxoreu 0.29693	2,000	60	74 ± 0.4	2.33 ± 0.05	0.812 ± 0.013	0.27 ± 0.05	0.190 ± 0.035	3,360 ± 619
Brazil, Otorere 0.36290	1,600 2,000	0.09 ± 0.01 0.65 ± 0.04	2.9 ± 0.3 3.0 ± 0.4	8.69 ± 1.27 8.99 ± 1.56	0.536 ± 0.088 0.562 ± 0.102	0.23 ± 0.01 0.10 ± 0.01	0.178 ± 0.009 0.186 ± 0.002	1,127 ± 34 4,532 ± 322
Central Africa 0.16070	2,000	106 ± 6	330 ± 2.6	0.79 ± 0.02	1.07 ± 0.020	0.60 ± 0.05	0.206 ± 0.014	834 ± 56
Africa 90706 0.15498	1,600 2,000	2.3 ± 0.1 42.8 ± 2.2	46 ± 1.2 89 ± 1.6	0.84 ± 0.06 0.93 ± 0.07	0.936 ± 0.041 0.780 ± 0.020	0.24 ± 0.02 0.13 ± 0.02	0.209 ± 0.010 0.235 ± 0.035	1,830 ± 85 4,570 ± 662
Africa 90706P* 0.17911	800 1,500 1,600 1,700 1,800 1,900	0.2 ± 0.01 1.1 ± 0.06 0.73 ± 0.04 22.9 ± 1.2 5.73 ± 0.3 0.00044	2.5 ± 0.5 9.0 ± 0.4 4.9 ± 0.5 120 ± 1.5 29 ± 0.8 11 ± 0.7	11.46 ± 2.82 3.507 ± 0.322 2.739 ± 0.67 1.002 ± 0.032 0.915 ± 0.13 9.75 ± 0.92	0.751 ± 0.187 0.453 ± 0.034 0.487 ± 0.089 0.507 ± 0.015 0.475 ± 0.025 —	0.41 ± 0.03 0.20 ± 0.02 0.04 ± 0.02 0.78 ± 0.05 0.09 ± 0.01 2.32 ± 0.15	0.192 ± 0.008 0.194 ± 0.031 — 0.203 ± 0.004 0.203 ± 0.029 0.187 ± 0.002	428 ± 13 953 ± 86 1,207 ± 700 580 ± 11 1,359 ± 192 303 ± 9
Air				9.800	0.029		0.1869	295.5

TABLE 1b Isotopic ratios of krypton in carbonados

SAMPLE	Temperature (°C)	^{84}Kr ($10^{-12} \text{ cm}^3 \text{ g}^{-1}$)	$^{78}\text{Kr}/^{84}\text{Kr}$	$^{80}\text{Kr}/^{84}\text{Kr}$	$^{82}\text{Kr}/^{84}\text{Kr}$	$^{83}\text{Kr}/^{84}\text{Kr}$	$^{86}\text{Kr}/^{84}\text{Kr}$
Brazil, Poxoreu	2,000	4.7 ± 0.1	0.005 ± 0.001	0.032 ± 0.002	0.1650 ± 0.005	0.2240 ± 0.007	1.5210 ± 0.042
Brazil, Otorere	1,600 2,000	7.1 ± 0.3 8.2 ± 0.2	0.006 ± 0.002 0.006 ± 0.001	0.039 ± 0.002 0.041 ± 0.002	0.1998 ± 0.008 0.1991 ± 0.004	0.1986 ± 0.008 0.2040 ± 0.005	0.3143 ± 0.013 0.3438 ± 0.007
Central Africa	2,000	150 ± 3	0.007 ± 0.001	0.037 ± 0.001	0.1836 ± 0.003	0.2098 ± 0.003	0.8510 ± 0.015
Africa 90706	1,600 2,000	7.4 ± 0.4 11 ± 0.9	0.006 ± 0.004 0.004 ± 0.001	0.038 ± 0.004 0.022 ± 0.003	0.1965 ± 0.011 0.1093 ± 0.012	0.2025 ± 0.012 0.2390 ± 0.018	0.5658 ± 0.030 3.0570 ± 0.182
Africa 90706P*	800 1,500 1,600 1,700 1,800 1,900	30 ± 0.5 16 ± 0.5 7.6 ± 0.4 53 ± 0.8 6.2 ± 0.4 8.4 ± 0.5	0.006 ± 0.001 0.008 ± 0.001 0.006 ± 0.001 0.006 ± 0.001 0.006 ± 0.001 0.006 ± 0.002	0.0404 ± 0.001 0.0397 ± 0.002 0.0388 ± 0.002 0.0380 ± 0.001 0.0349 ± 0.003 0.0383 ± 0.003	0.2050 ± 0.004 0.1995 ± 0.007 0.2017 ± 0.011 0.1925 ± 0.003 0.1795 ± 0.012 0.1973 ± 0.013	0.2009 ± 0.003 0.2023 ± 0.008 0.1993 ± 0.010 0.2042 ± 0.003 0.2123 ± 0.015 0.2031 ± 0.013	0.3086 ± 0.010 0.3335 ± 0.011 0.3670 ± 0.018 0.5810 ± 0.008 1.0612 ± 0.062 0.3099 ± 0.019
Air			0.0061	0.0396	0.2022	0.2016	0.3055

TABLE 1c Isotopic ratios of xenon in carbonados

SAMPLE	Temperature (°C)	^{130}Xe ($10^{-12} \text{ cm}^3 \text{ g}^{-1}$)	$^{124}\text{Xe}/^{130}\text{Xe}$	$^{126}\text{Xe}/^{130}\text{Xe}$	$^{128}\text{Xe}/^{130}\text{Xe}$	$^{129}\text{Xe}/^{130}\text{Xe}$	$^{131}\text{Xe}/^{130}\text{Xe}$	$^{132}\text{Xe}/^{130}\text{Xe}$	$^{134}\text{Xe}/^{130}\text{Xe}$	$^{136}\text{Xe}/^{130}\text{Xe}$
Brazil, Poxoreu	2,000	0.23 ± 0.02	0.043 ± 0.033	0.022 ± 0.022	0.462 ± 0.049	6.53 ± 0.42	33.8 ± 2.07	200.8 ± 12.2	273.3 ± 16.6	329.5 ± 20.1
Brazil, Otorere	1,600 2,000	0.14 ± 0.01 0.21 ± 0.01	0.009 ± 0.008 0.019 ± 0.012	0.005 ± 0.005 0.071 ± 0.058	0.497 ± 0.085 0.459 ± 0.033	6.35 ± 0.42 6.59 ± 0.21	5.38 ± 0.31 5.83 ± 0.22	7.26 ± 0.4 10.97 ± 0.3	3.46 ± 0.24 8.93 ± 0.24	3.21 ± 0.2 9.76 ± 0.4
Central Africa	2,000	0.68 ± 0.14	0.054 ± 0.023	0.034 ± 0.015	0.622 ± 0.140	7.66 ± 1.76	47.5 ± 9.84	280.2 ± 58	395.6 ± 82	476.6 ± 99
Africa 90706	1,600 2,000	0.14 ± 0.01 0.21 ± 0.02	0.031 ± 0.031 0.030 ± 0.030	0.039 ± 0.040 0.046 ± 0.046	0.464 ± 0.124 0.471 ± 0.086	6.23 ± 0.56 7.15 ± 0.90	11.5 ± 1.05 68.6 ± 7.57	49.3 ± 4.3 432.3 ± 47.6	64.93 ± 5.7 606.8 ± 67.4	77.2 ± 6.9 730.5 ± 81.4
Africa 90706P*	800 1,500 1,600 1,700 1,800 1,900	0.77 ± 0.03 0.41 ± 0.02 0.23 ± 0.01 1.4 ± 0.03 0.25 ± 0.01 0.21 ± 0.01	0.022 ± 0.007 0.026 ± 0.026 0.051 ± 0.048 0.025 ± 0.004 0.038 ± 0.030 0.028 ± 0.028	0.036 ± 0.012 0.018 ± 0.018 0.070 ± 0.042 0.024 ± 0.005 0.035 ± 0.035 —	0.461 ± 0.023 0.454 ± 0.041 0.513 ± 0.064 0.471 ± 0.020 0.488 ± 0.060 0.453 ± 0.064	6.46 ± 0.22 6.39 ± 0.32 6.3 ± 0.43 6.43 ± 0.15 6.69 ± 0.34 6.28 ± 0.45	5.24 ± 0.17 5.50 ± 0.27 6.04 ± 0.40 8.51 ± 0.18 16.5 ± 0.73 9.39 ± 0.63	6.79 ± 0.21 9.23 ± 0.44 12.3 ± 0.73 29.3 ± 0.59 82.9 ± 3.51 34.9 ± 2.21	2.95 ± 0.12 6.55 ± 0.33 11.11 ± 0.74 34.98 ± 0.85 111.3 ± 5.04 43.42 ± 2.88	2.62 ± 0.1 6.86 ± 0.3 12.22 ± 0.7 41.21 ± 1.0 133.1 ± 6.0 51.69 ± 3.4
Air			0.024	0.022	0.471	6.45	5.198	6.600	2.562	2.177

Isotopic measurements were made separately for He, Ne, Ar and Kr + Xe with the use of liquid nitrogen cold traps and a helium cryopump. Noble gas amounts were measured from their peak heights in the mass spectrum. Errors in isotopic ratios include 1σ in mass spectrometry and 20% of procedural hot blanks which were 5×10^{-10} , 5×10^{-13} , 10^{-11} , 7×10^{-14} and 4×10^{-14} (all quantities in cm^3) for ^4He , ^{22}Ne , ^{36}Ar , ^{84}Kr and ^{132}Xe respectively at 2,000 °C. Isotopic ratios of Xe and Kr are corrected for mass discrimination: 0.5% per AMU for Xe, 1.0% per AMU for Kr. No discrimination correction was made for Ne and Ar, as the average $^{20}\text{Ne}/^{22}\text{Ne}$ and $^{38}\text{Ar}/^{36}\text{Ar}$ values for repeated standard air samples are identical with the air ratios within 0.5%.

* A diamond from same batch as sample 90706, pulverized (to $\leq 10 \mu\text{m}$) and thoroughly cleaned with hot HF and HNO_3 to remove possible contaminants.

^{86}Kr and ^4He (values in brackets) that could be produced in 4.5 Gyr from the uranium contained in each sample. By comparing the calculated values with the observed amounts, it is obvious that most of the fission Xe, Kr and ^4He cannot have been produced *in situ*, and we must conclude that these noble gases were imported. As the fission Xe and Kr and the ^4He emitted from decaying U and Th have fairly high energies, they can

easily be implanted from surrounding materials that are in close contact with the micro-diamonds; the range for ^{238}U fission particles¹³ is $\sim 20 \mu\text{m}$ and the diameters of individual micro-diamonds in a carbonado are at most a few micrometres. If the surrounding materials have a high uranium content (more than a few hundred p.p.m.), the observed amount of the excess noble gases can be implanted into the carbonados in a geologically

TABLE 2

SAMPLE	$\delta^{13}\text{C}$	U (p.p.m.)	^4He ($10^{-3}\text{ cm}^3\text{ g}^{-1}$)	$(^{21}\text{Ne})_{\text{excess}}$ ($10^{-12}\text{ cm}^3\text{ g}^{-1}$)	$(^{86}\text{Kr})_{\text{fission}}$ ($10^{-12}\text{ cm}^3\text{ g}^{-1}$)	$(^{136}\text{Xe})_{\text{fission}}$ ($10^{-12}\text{ cm}^3\text{ g}^{-1}$)
Brazil (Poxoreu)	not measured	2.3 ± 0.03	60 (3.9)	5.9	6 (1.1)	75.8 (7.6)
Brazil (Otorere)	-27.4, -27.4	0.67 ± 0.02	0.73 (1.1)	3.3	0.33 (0.33)	1.7 (2.2)
Central Africa	-29.6, -29.6	5.6 ± 0.16	106 (9.5)	350	84.8 (2.8)	322.6 (18.5)
Africa 90706	-23.9	7.8 ± 0.14	45 (13.3)	112	33.8 (3.9)	164 (25.7)

Numbers in brackets are the amounts of noble gas calculated for $t=4.5 \times 10^9$ yr from the uranium contents in column 3. To calculate ^4He and $(^{21}\text{Ne})_{\text{excess}}$, we assumed U/Th=3.3. $\delta^{13}\text{C}$ was measured on the graphite residues after noble gas extraction and represented relative to the PDB standard normalizing to $\delta^{13}\text{C}_{\text{PDB}} = -1.06\%$ for NBS-20. $\delta^{13}\text{C} = [(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}} - 1] \times 1,000$. Uranium content was measured by a neutron activation method using a low energy photon spectrometer. A sample in a cadmium capsule was irradiated by a thermal neutron flux of $1.6 \times 10^{12}\text{ n cm}^{-2}\text{ s}^{-1}$ with Cd(Au) ratio of 3.

reasonable time, say in 1 Gyr. Such a uranium-enriched environment is most likely to be found in the crust, favouring the crustal origin of carbonados. Coals are a good candidate for the uranium source, as they are known to enrich uranium¹⁴. Implantation can also explain the excess Ne isotopes, as Ne isotopes produced by the Wetherill reactions have recoil energies that are high enough for implantation (for example, 0.124 MeV for $^{18}\text{O}(\alpha, n)^{21}\text{Ne}$ and 0.189 MeV for $^{19}\text{F}(\alpha, n)^{22}\text{Ne}$).

Figure 2 shows thermal release patterns for ^4He , excess ^{22}Ne , ^{86}Kr and ^{136}Xe , and non-nucleogenic components ^{20}Ne , ^{36}Ar , ^{80}Kr and ^{130}Xe produced on step-heating the sample 90706P. The thermal release patterns of ^4He , ^{22}Ne , ^{86}Kr and ^{136}Xe are almost identical, but they are distinctly different from those for non-nucleogenic components. The difference must arise because the non-nucleogenic components originate from trapped gases, whereas the nucleogenic components were implanted. As seen in Fig. 2, ~60% of ^{36}Ar was degassed at the highest temperature, where graphitization took place. This fraction has an $^{40}\text{Ar}/^{36}\text{Ar}$ ratio identical to that of atmospheric argon (see Table 1). The fact that the gas release occurred at the graphitization temperature indicates that this fraction was trapped during the crystallization of diamonds. The lower-temperature fractions have $^{40}\text{Ar}/^{36}\text{Ar}$ ratios considerably higher than atmospheric and are likely to represent *in situ* radiogenic components; judging from the high uranium content, the diamond must contain some potassium. Additional evidence for trapped atmospheric noble gases can be seen in Ne, Kr and Xe isotopic compositions in this experiment. The highest-temperature fractions show

$^{20}\text{Ne}/^{22}\text{Ne}$ and Kr isotope ratios indistinguishable from the atmospheric values, but the lower-temperature fractions (except for the lowest, which has a very large error) show distinctly nucleogenic components (see Table 1): this release pattern is very similar to that for ^{36}Ar . For Xe, all the temperature fractions show nearly the same $^{129}\text{Xe}/^{130}\text{Xe}$ ratios. These are identical with the atmospheric ratio, but distinctly different from the mantle component, which is characterized by an excess ^{129}Xe .

The amounts of the trapped components, for example in sample 90706, are $\sim 1.2 \times 10^{-10}\text{ cm}^3\text{ g}^{-1}$ for ^{20}Ne , $3.7 \times 10^{-10}\text{ cm}^3\text{ g}^{-1}$ for ^{36}Ar and $3.5 \times 10^{-13}\text{ cm}^3\text{ g}^{-1}$ for ^{130}Xe . These amounts are more than those commonly observed for mid-ocean-ridge basalt glasses¹⁵. As no mantle-derived materials have been reported to contain such large amounts of atmospheric noble gases, this seems to leave the crust as the only possible place for the crystallization of the carbonados. One might argue that the carbonados were formed in the mantle and the enormous amount of fission Xe is due to uranium-rich secondary minerals which replaced the original inclusions in the diamonds after emplacement of the diamonds in the crust. We consider, however, that the very tightly trapped atmosphere noble gases in the carbonados rule out this possibility, as discussed above.

All four carbonados studied show the same characteristic features: unsupported fission Xe and Kr; nucleogenic Ne and radiogenic ^4He ; very light $\delta^{13}\text{C}$ typical of a biogenic origin (no measurement for Brazil Poxoreu); and tightly trapped atmospheric noble gases. Although four samples are too few to warrant a final conclusion, it is difficult to argue that the common features for four samples from different localities are merely coincidence. We are particularly impressed by the large amount of unsupported fission Xe and Kr, which indicates the intimate association of uranium atoms with carbonado microcrystals for a considerable time, and suggests that uranium may have played an important part in forming the carbonado (as was speculated previously⁸). The observation¹⁶ that irradiation of a coalified log by energetic particles resulted in its dehydrogenation to form diamond-like clusters of carbon atoms may be relevant to carbonado formation. □

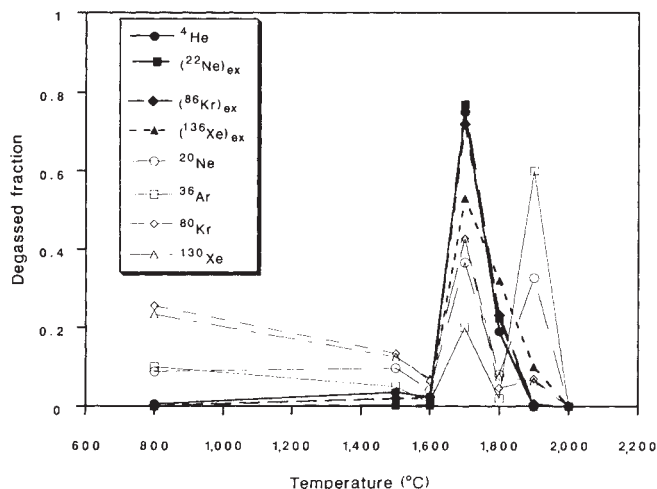


FIG. 2 Step-heating degassing of noble gases from a pulverized carbonado. Sample, Africa 90706P. Thick lines with solid symbols represent implanted nucleogenic components, thin lines with hollow symbols represent trapped atmospheric components. Note that the former components were degassed essentially at 1,700 °C, whereas the latter have second degassing peaks at 1,900 °C where graphitization took place.

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Lack of regional specificity for connections formed between thalamus and cortex in coculture

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THE mammalian cerebral cortex consists of many structurally¹ and functionally² specialized areas, with characteristic input from particular nuclei of the thalamus. Some localized external influence, such as the arrival of fibres from the appropriate thalamic nucleus before or around the time of birth³⁻⁶, could trigger the emergence of committed cortical fields from an undifferentiated 'protocortex'^{7,8}. The guidance of axons from each thalamic nucleus to its appropriate target area in the cortex could, then, be crucial in the regulation of cortical differentiation. Recently, Yamamoto *et al.*⁹ and Bolz *et al.*¹⁰ have demonstrated that cocultured explants of rat lateral geniculate nucleus and visual cortex can form layer-specific interconnections. We have now tested the possibility that each cortical area exerts a selective trophic influence on axons from its appropriate thalamic nucleus, and vice versa, by coculturing explants of different regions of the thalamus and cortex taken at various stages of development.

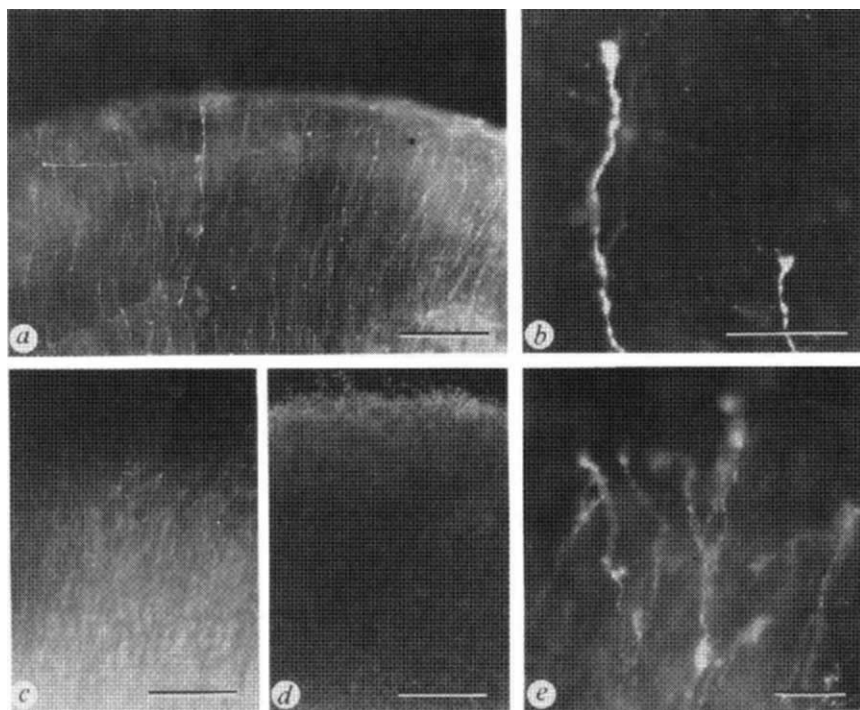
FIG. 1 Dark-field micrographs under epifluorescent illumination, showing Dil-labelled axons from an E16 LGN explant invading cortical slices after 4 days in culture. *a*, In P0 occipital cortex, axons are seen running radially or obliquely up through the cortical plate to the marginal zone, where some have turned laterally to run over or beneath the pial surface. One axon (far right) has turned 90° and run horizontally more than 1.5 mm: at its tip is a growth cone. Scale bar, 0.25 mm. *b*, High-power view of growth cones at the tips of axons below the cortical surface in the same coculture. Scale bar, 50 µm. *c*, With P6 occipital cortex, axons from an E16 LGN explant have invaded densely and most are branching and losing their growth cones about 300 µm below the pial surface. Scale bar, 0.25 mm. *d*, The same as *c*, but viewed under ultraviolet illumination to reveal the pial surface, after staining with bisbenzamide. Scale bar, 0.25 mm. *e*, High-power view from *c*, showing individual axons branching and apparently terminating. Scale bar, 50 µm.

METHODS. Fetal Sprague-Dawley rats were obtained from time-mated females by caesarian section under pentobarbital anaesthesia (100 mg kg⁻¹, intraperitoneal); surgery on post-natal animals was performed under hypothermia or pentobarbital anaesthesia. Coronal slices of neocortex or hippocampus, or sagittal slices of cerebellum, 350–400 µm thick, cut with a hand-held tissue chopper, were placed on collagen-coated microporous membranes in stationary Costar Transwell-COL culture chambers with N2 hormone-supplemented serum-free medium just covering the explant, at 36 °C in 5% CO₂. Such slices survive well, preserving their organotypic characteristics, and remain about 150–200 µm thick even after many weeks, rather than collapsing to virtual monolayers as with the roller-tube method¹⁴. Explants of E15–17 fetal thalamus, as small as 0.5 × 0.5 × 0.3 mm, were dissected from the dorsolateral aspect of the posterior diencephalon (LGN), from an equivalent position more anterior in the thalamus (corresponding to nuclei that become connected to frontal cortical fields), or from other parts of the developing thalamus. To learn how to recognize the position of the LGN and hence other parts of the thalamus reliably from surface landmarks, we started by exposing several E16 fetal rats by hysterotomy and injected one eye with horseradish peroxidase (HRP). These fetuses were delivered by caesarian section a day later and sections of the brains were processed

Although thalamo-cortical and cortico-thalamic connections formed *in vitro* can be remarkably normal in many respects, they lack regional specificity.

According to Lund and Mustari³, axons from the lateral geniculate nucleus (LGN) start to invade the cortical plate in the rat around the time of birth (gestation is 21–22 days). We began, then, by combining slices of rat occipital (putative visual) cortex, taken near the time of birth, from embryonic day 20 to postnatal day 3 (E20–P3), with small explants of the LGN taken from fetal rats at E15–17, when dense outgrowth of axons occurs *in vivo*^{3,11}, just after the formation of separate nuclei. We used serum-free medium for organotypic culture¹² and briefly prelabelled the LGN explant in a solution of the lipophilic carbocyanine dye, DiI¹³, which enabled us to follow the outgrowth of labelled axons by epifluorescence microscopy. Thalamic axons streamed out in the direction of the cortical slice, entered the white matter and ran radially through the cortical plate at up to 1 mm per day. The general pattern of innervation appeared to be surprisingly normal, except that the axons did not terminate in the middle layers of the cortex but reached the marginal zone after about 3 days in culture and started to grow laterally, under and on the pial surface (Fig. 1*a*). At the ends of the axons were expanded growth cones (Fig. 1*b*).

We wondered whether this failure to terminate normally reflects the incomplete development of cortical lamination at the age at which the slices were taken: on the day of birth, cells



for HRP histochemistry to reveal the terminal fields of optic nerve axons in the LGN. We proved the reliability of our identification of the major divisions of the thalamus from surface landmarks by cutting, staining and examining sections of the remaining part of the diencephalon to be sure that we had removed the desired region for culture. One of the explants in each coculture group was prelabelled by brief immersion in a solution of DiI in alcohol and dimethyl sulphoxide before rinsing in Hank's balanced-salt solution. This caused minute crystals of DiI to condense on the surface of the explant: most of the axons growing out were intensely labelled with the fluorescent dye for a period of about 5 days (after which the label became concentrated back in the cell body membranes, presumably because of membrane recycling). After fixing the coculture in 4% paraformaldehyde, we examined the labelled axons with fluorescence microscopy. Counterstaining with bisbenzamide (10 min in 2.5 µg ml⁻¹ solution in phosphate buffer) allowed us to visualize the pial surface under ultraviolet illumination. This report is based on more than 400 successful organotypic cocultures.

that will form layer 4, the principal target of thalamic fibres, are gathering under the marginal zone or even still migrating through the plate³. We therefore cocultured fetal LGN with older slices of occipital cortex (P5–11), when the lamination of the cortex is more mature. Within 4 days, many invading thalamic axons had branched locally, lost their growth cones and apparently terminated some 300 μm below the pial surface (Fig. 1c). As *in vivo*, some axons extended to the marginal zone.

It seems, then, that occipital cortex can exert a trophic influence on the LGN in culture (or is at least permissive for axon invasion) and that, sometime between P3 and P5, it generates a signal that causes most thalamic fibres to stop below the marginal zone. When we placed E16 LGN explants against the cleaned pial surface of older slices of cortex, axons readily grew in through the supragranular layers and again tended to termin-

ate in the middle of the cortical plate. Therefore this expressed signal is not one that simply discourages growth in the upper layers: it seems likely that around P4, neurons of layer 4, having reached their correct position, express a 'stop signal', which encourages thalamic axons to arborize and terminate.

We adopted a 'choice' protocol to examine the preferences of thalamic explants for various target tissues: individual thalamic explants were flanked by two slices dissected from different regions of an individual brain, and innervation of the two was compared. Figure 2a–c shows the results of culturing E16 LGN for 5 days with a choice between P6 occipital cortex and some other nervous tissue. The most dramatic result is the contrast between the florid innervation of occipital cortex and the virtual avoidance of a slice of P6 cerebellum (Fig. 2a). In 37 cocultures involving LGN and cerebellum (from P1 to P8) we have seen only a few axons actually penetrating cerebellar slices.

Given the choice between occipital cortex and P6 hippocampus (Fig. 2b), LGN explants showed little preference, but axons grew towards the surface of the hippocampus and did not terminate in a particular layer, as in the cortex at this age. This implies that the hippocampus, a region of allocortex, may exert a general influence similar to that of neocortex but has no equivalent of the 'stop signal' expressed by cortical layer 4.

To test whether different cortical areas selectively attract fibres from their particular thalamic nuclei, we cultured explants of LGN or other regions of the developing thalamus with a choice between appropriate and inappropriate cortical slices. The result was unequivocal: the pattern of innervation was indistinguishable whatever regions of thalamus and cortex were combined (Fig. 2c). Explants of posterior, anterior and more ventral thalamus sent axons in a similar pattern into occipital, frontal or any other region of cortex. If these results apply *in vivo*, the developing cortex encourages thalamic axons to invade and terminate with laminar specificity; but their guidance to the correct cortical area is unlikely to depend on the remote effect of regionally specific trophic influences of cortex on thalamus.

Recent studies on cat^{4,15} and rat^{11,16,17} show that the earliest postmitotic cortical cells, which form the subplate below the cortical plate proper, send out pioneering axons towards the thalamus at a very early stage, before cells of the main layers of the cortex have migrated into position and before the arrival of thalamic fibres. These early corticofugal axons could guide their thalamic counterparts to their correct cortical areas. It occurred to us that each thalamic nucleus could exert a specific trophic influence on subplate axons from the appropriate region of the cortex and that this might be the essential mechanism underlying the establishment of topographic cortical innervation.

We tested this idea by culturing thalamic explants with DiI-labelled slices of embryonic cortex taken as early as E14, when the first neurons of the cortical plate proper are just being born^{3,17,18} but the subplate cells are already in place. Figure 3 summarizes the results. Embryonic cortex did send axons into thalamic explants, but again there was no obvious regional preference. Any zone of embryonic cortex innervated equally any region of developing thalamus, even when presented with a choice of appropriate and inappropriate regions. Because these cortical explants included the germinal ventricular zone, it is conceivable that the birth and migration of neurons continued in culture. Therefore some of the corticofugal axons seen after 4 days *in vitro* could have come from cells of the lower layers of the cortical plate proper. But because invading axons were seen after only 1 day in culture and they had grown as far as 3 mm after 4 days (Fig. 3), it seems likely that most of them come from subplate cells.

We conclude that the remarkable regional specificity of thalamo-cortical interconnection is not achieved through highly selective target chemotropism. Indeed, our work on the pattern of early cortico-thalamic and thalamo-cortical projections in the

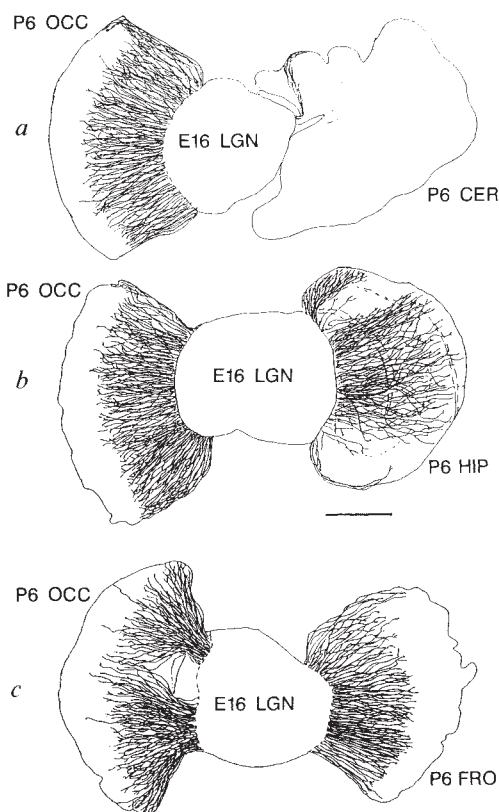


FIG. 2 Results of the forced choice experiment in which each thalamic explant was cultured next to two different slices. Camera-lucida drawings show DiI-labelled thalamic axons after 5 days in culture. *a*, Confronted with P6 occipital cortex (OCC) and cerebellum (CER), E16 LGN innervation was almost entirely restricted to the slice of neocortex, in which a few axons reached the marginal zone but most terminated in what appeared to be layer 4, about 300 μm below the surface. Of the few axons that sprouted towards the cerebellar slice most ran around one border. *b*, When cultured with P6 occipital cortex and hippocampus (HIP), the density of innervation from E16 LGN was similar, but in the hippocampus the axons did not terminate in a distinct zone and mainly extended towards the surface. Scale bar, 1 mm. In this case, the hippocampal explant was a coronal slice containing part of the CA1–CA3 segments and part of the dentate gyrus (closest to the LGN explant). Interrupted lines indicate laminar boundaries in the hippocampus, visible under ultraviolet illumination after counterstaining with bisbenzamide. In other cocultures, different regions of the hippocampus and different orientations of the explant were used, but the pattern of thalamic innervation was always similar, with no preference for any particular segment of the hippocampus. *c*, With P6 occipital and frontal cortex (FRO), geniculate axons invaded both slices densely with very similar patterns. In both cases a few axons reached the marginal zone but most terminated around layer 4. The examples shown here were chosen as representative from 234 cocultures of fetal thalamus and perinatal neocortex, 37 of thalamus and cerebellum, and 36 of thalamus and hippocampus.

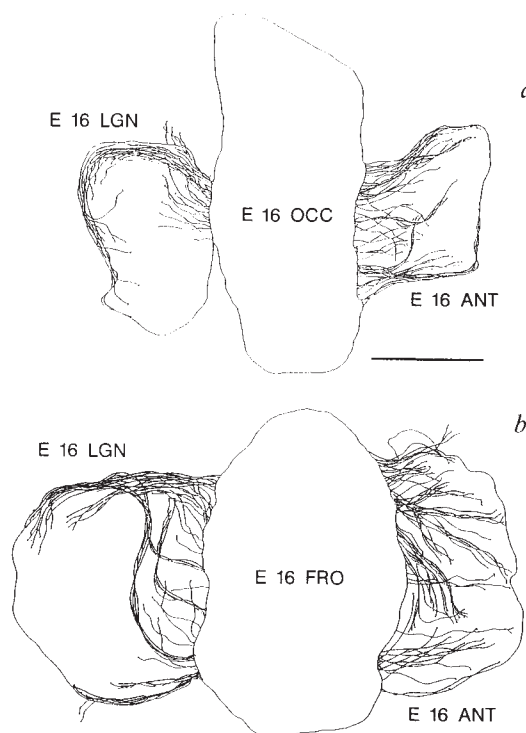


FIG. 3 Camera-lucida drawings of axon outgrowth from E16 cortical explants, pre-labelled with Dil, cultured for 4 days with a choice of thalamic explants of the same age. *a*, Occipital cortex (OCC) sends axons (presumably mainly if not exclusively from subplate cells) into LGN and anterior thalamus (ANT). *b*, Frontal cortex (FRO) also innervates LGN and anterior thalamus very similarly. Scale bar, 1 mm. In these experiments, each pair of thalamic explants was dissected from the same side of an individual brain, to ensure that they consisted of different thalamic nuclei. The pattern of cortical innervation of thalamic explants was more variable than that of thalamo-cortical ingrowth *in vitro*. In some cases, cortical axons grew fairly uniformly, but in others they tended to form fascicles or to favour certain segments of the thalamic explant. These examples, selected from 122 cocultures, involving E15–E16 cortex and thalamus, illustrate the range of variation, which did not correlate with the sites of origin of the explants.

rat^{11,16} suggests that the two outgrowths are synchronized, that each pioneers the pathway through its own segment of the brain as a tightly organized axon array, that they meet each other near the primitive internal capsule, and that each provides contact guidance for the other over the distal part of its trajectory. The key, then, to selective afferent innervation of the cortex (which may itself underlie regional differentiation) could lie in some fairly simple property of the extracellular environment between thalamus and cortex, determining the direction of growth of both sets of axons towards the primitive internal capsule. That, combined with a strict sequence of outgrowth and a tendency of fibres not to cross their neighbours, might be enough to establish the two ordered patterns of pioneers, which could then guide each other home. □

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Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid

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THE fundus of the guinea-pig stomach actively dilates in response to low increases in intragastric pressure¹. This physiological response, now called adaptive relaxation^{2,3}, accommodates the intake of liquid or food. It is independent of external innervation, resistant to ganglion blockade, but reflex in origin. The nerves involved are neither adrenergic nor cholinergic in nature. Non-adrenergic, non-cholinergic (NANC) nerves have now been recognized in many parts of the gastrointestinal tract⁴ and have recently been linked with release of nitric oxide (NO) on electrical stimulation^{5–7}. Here we show that adaptive relaxation in isolated stomach of the guinea pig is mediated by a NANC neurotransmitter substance indistinguishable from NO derived from L-arginine⁸. This is substantiated by inhibition of adaptive relaxation by N^G-monomethyl-L-arginine or N^ω-nitro-L-arginine methyl ester, both inhibitors of NO synthesis^{9,10}, and by methylene blue, an inhibitor of soluble guanylate cyclase¹¹. There are two distinct neuronal pathways signalling NO-dependent adaptive relaxation, as evidenced by tetrodotoxin sensitivity. The first is a local reflex arc, the afferent fibres of which sense changes in intragastric pressure. The second is stimulated by an agonist for ganglionic nicotinic receptors. Thus, the functional significance of NO release from NANC nerves in the stomach is to bring about adaptive relaxation through a reflex response to increases in intragastric pressure.

The guinea-pig stomach was emptied of luminal contents and the oesophagus ligated close to the stomach. The pyloric end was cannulated and connected to a large reservoir containing oxygenated Krebs's solution. Increments in gastric pressure increased gastric volume up to a point where gastric volume increased sharply without any further increase in the applied pressure (Fig. 1). This burst-like adaptive relaxation of the stomach occurred at gastric pressures of 7.1 ± 1.5 cm H₂O ($n = 31$ stomachs) and allowed for a substantial increase in gastric volume ($47.7 \pm 7.3\%$ of the total gastric volume, $n = 31$). Incubation of the stomach (luminally and extraluminally) with tetrodotoxin ($2 \mu\text{M}$, for 30 min) completely eliminated the adaptive response ($n = 3$), demonstrating that this pressure-induced adaptive relaxation was mediated by nerves. Incubation of the stomach with guanethidine ($5 \mu\text{M}$) to inhibit adrenergic nerve function, together with atropine ($3 \mu\text{M}$) to inhibit cholinergic (muscarinic) function, did not inhibit pressure-induced fundal relaxations ($n = 5$), showing that this response was mediated by NANC nerves⁴.

NO is released from NANC nerves stimulated by an electrical

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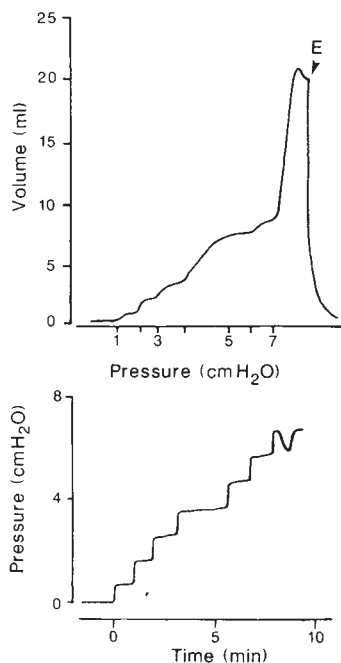


FIG. 1 A typical trace demonstrating pressure-induced adaptive relaxation of isolated stomach from the guinea-pig. Isolated stomachs of male Hartley guinea-pigs (250–350 g) were placed into organ baths (450 ml, 37 °C) containing Krebs' solution¹ and the pyloric cannula attached to a large reservoir (2 l) containing oxygenated (95% O₂/5% CO₂) Krebs' solution and sealed by a float recorder. For a typical control record, the level of Krebs' solution in the reservoir was adjusted to that of the stomach cavity so that the pressure was zero and no fluid entered the stomach. The reservoir was then elevated stepwise (in 1-cm increments) and Krebs' solution entered the stomach as the pressure increased (1–20 cm H₂O). Increases in gastric pressure induced filling of the stomach until a pressure was reached (7 cm of H₂O in this experiment) at which gastric volume increased greatly and gastric pressure remained low. After adaptive relaxation, the stomach was emptied (E). Adaptive relaxation could be induced reproducibly many times throughout the duration of the experiment (4–6 h). Gastric pressure was monitored by a Satham pressure transducer and the corresponding gastric volume was measured with a float recorder attached to a Harvard isotonic transducer. Changes in pressure and volume were displayed on a Graphtec WR3101 recorder. This trace is representative of 31 experiments.

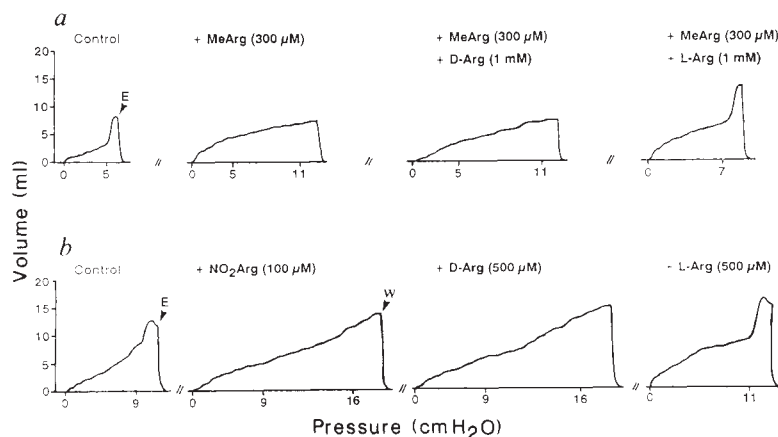
field or by agonists acting at ganglionic nicotinic receptors in the canine ileocolonic junction⁵, rat anococcygeus⁶ and rat stomach fundus⁷. Therefore, we tested the effects of NO synthesis inhibitors on pressure-induced adaptive relaxations of the guinea pig stomach. Incubation of the stomach for 30 min with *N*^G-monomethyl-L-arginine (MeArg; 300 μ M; $n=3$) or *N*^w-nitro-L-arginine methyl ester (NO₂Arg; 100 μ M; $n=9$) abolished the adaptive relaxation (Fig. 2). The inhibitory effect of MeArg was partially reversed by co-incubation with L-arginine (1 mM; $n=3$) but not by D-arginine (1 mM; $n=3$). Co-incubation of L- or D-arginine (1–5 mM) did not modify the inhibitory action of NO₂Arg. Washing out NO₂Arg followed by L-arginine (0.5 mM; $n=4$) but not D-arginine (0.5 mM; $n=8$) reversed the inhibitory effect, suggesting that NO₂Arg has a higher affinity for gastric NO synthase than does MeArg. Incubation of the stomach with L-arginine alone (0.5–2 mM) did not modify the pressure-volume relationship ($n=3$), indicating that the supply of L-arginine for NO synthesis was not rate-limiting.

NO relaxes various vascular and non-vascular tissues through activation of soluble guanylate cyclase, thereby elevating cyclic GMP levels^{11,12}. Incubation of the stomach (45–60 min) with methylene blue (20–40 μ M), an inhibitor of soluble guanylate cyclase¹³, also inhibited adaptive relaxation ($n=4$) supporting our conclusion that NO is the mediator of fundal relaxation induced by pressure increases in our experiments or by electrical stimulation¹.

To understand the nature of pressure-induced neuronal generation of NO, we examined whether activation or antagonism of ganglionic nicotinic receptors could influence adaptive relaxation. In the presence of guanethidine (5 μ M) and atropine (3 μ M), stimulation of nicotinic receptors in ganglia with 1,1-dimethylphenyl 4-piperazinium (DMPP, 1–10 μ M) elicited concentration-dependent relaxations of the stomach. These relaxations were abrogated by incubation with MeArg (300 μ M; $n=3$; Fig. 3) or NO₂Arg (100 μ M; $n=3$). Co-incubation or washout with L- but not D-arginine (0.5–2 mM, $n=3$ for L- and D-arginine) partially restored the stimulation by DMPP. Furthermore, the effect of DMPP was antagonized by tetrodotoxin (2 μ M, $n=3$) or by the ganglionic nicotinic receptor antagonist, hexamethonium (0.5 mM; $n=3$). However, hexamethonium did not block the pressure-induced adaptive relaxation response ($n=3$), as described previously¹.

These data demonstrate the existence of at least two different neuronal pathways resulting in NO-mediated adaptive relaxation of the stomach. One is a local reflex arc activated by changes in intragastric pressure which is resistant to blockade of cholinergic ganglia; the other is activated by an agonist for ganglionic nicotinic receptors. Our experiments do not distinguish whether NO is the mediator of ganglionic transmission as well as of fundal relaxation. Evidence demonstrating the generation of nitrogen oxides by neural structures^{14–16} and the immunolocalization of NO synthase in nerve fibres and cell bodies of the

FIG. 2 Inhibition of NO synthesis prevents adaptive relaxation in isolated stomach from the guinea-pig. Incubation of the stomach (luminally and extraluminally for 30 min) with MeArg (300 μ M; a) or NO₂Arg (100 μ M; b) inhibits the adaptive relaxation in response to pressure. Co-incubation (a) or washout (b) with L- but not D-arginine partially reverses the inhibitory effect. E, Emptying of the stomach; W, washout of NO₂Arg from the stomach. In time control experiments, the inhibitory effect of NO₂Arg persisted for 3–4 h after washout of the stomach with Krebs' solution only ($n=3$). This trace is representative of 3–9 experiments.



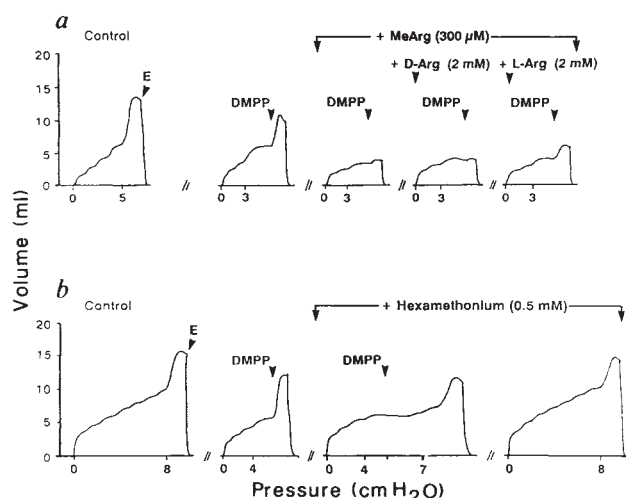


FIG. 3 DMPP (10 μ M) elicits adaptive relaxation of the stomach which is blocked by MeArg (300 μ M; a) or hexamethonium (0.5 mM; b), but hexamethonium does not prevent the pressure-induced adaptive relaxation (b). Before observing responses to DMPP, at least three control curves were done to find the pressure needed to elicit the adaptive response. Gastric pressure was then raised to a level just below that needed for an adaptive response, and DMPP added extraluminally. All experiments were done with guanethidine (5 μ M) and atropine (3 μ M) in the Krebs' solution. E, Emptying of the stomach. This trace is representative of 3–5 experiments.

myenteric plexus¹⁷ supports the role of NO as a mediator of intercellular communication in the gastrointestinal tract. Furthermore, inhibition of NO formation also blocks the smooth muscle relaxing activity of other putative NANC transmitters, such as vasoactive intestinal polypeptide, adenosine 5'-triphosphate or γ -aminobutyric acid, suggesting that NO is the final

mediator of the effects of such agents^{7,18}. Thus, adaptive relaxation of the stomach *in vivo* involves both local and peripheral (vagal) neural mechanisms, so it is likely that NO is the final common mediator of gastric smooth muscle relaxation elicited by either source of input. □

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Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines

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SEVERAL viral and bacterial live recombinant vaccine vehicles are being developed to produce a new generation of vaccines against a broad spectrum of infectious diseases¹. The human tuberculosis vaccine *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)² has features that make it a particularly attractive live recombinant vaccine vehicle. BCG and other mycobacteria are highly effective adjuvants, and the immune response to mycobacteria has been studied extensively. With nearly two billion immunizations, BCG has a long record of safe use in man^{3,4}. It is one of the few vaccines that can be given at birth, it engenders long-lived immune responses with only a single dose, and there is a worldwide distribution network with experience in BCG vaccination. Recently developed molecular genetic tools and methods for mycobacteria have provided the means to introduce foreign genes into BCG^{5–8}. Here we report that a variety of human immunodeficiency virus type 1 polypeptides can be expressed in BCG recombinants under the control of the mycobacterial hsp70 promoter and that the foreign polypeptides produced in BCG can induce antibody and T-cell

responses. These results demonstrate that BCG can be used as a live recombinant vaccine vehicle to induce immune responses to pathogen proteins produced by the bacillus.

We investigated whether three different human immunodeficiency virus type 1 (HIV-1) genes could be expressed in BCG. The mycobacterial heat-shock protein hsp70 promoter and translation start site was employed to direct the expression of foreign genes. These regulatory elements were selected because they are among the most powerful in bacteria⁹ and because hsp70 synthesis is induced to very high levels during phagocytosis of some bacteria by macrophage¹⁰. The heat-shock protein promoters also normally control the expression of proteins that are dominant antigens in the immune response to mycobacterial infection^{11–13}. DNA fragments encoding HIV-1 Gag, Pol and Env precursors were fused to the mycobacterial hsp70 promoter and translation start site so that they precisely replaced the hsp70 coding sequences, and the hybrid genes were inserted into the BCG autonomous replicating plasmid vector pYUB12⁶ (Fig. 1a). The recombinant plasmids were introduced into the Pasteur strain of *M. bovis* BCG by electroporation⁶.

The BCG recombinants produced HIV Gag, Pol or Env polypeptides when lysates of the bacteria were examined by western blotting using serum from an HIV-1 positive individual (Fig. 1b). A Gag polypeptide (relative molecular mass 52,000; M_r 52K) was present in lysates of BCG cells containing the 1.53-kilobase (kb) gag gene, a 92K Pol polypeptide was in BCG carrying the 2.76-kb pol gene, and an 85K Env polypeptide was in BCG containing the 2.56-kb env gene. The apparent M_r of each polypeptide was consistent with that predicted by the gene sequence in the absence of post-translation modification (Gag, 512 amino acids; Pol, 922 amino acids; Env, 856 amino acids).

TABLE 1 Production of γ -interferon (pg ml^{-1}) by spleen cells stimulated with HIV Gag peptides

Injection	HIV-1 Gag peptides (residues)						Control peptides
	1-93	86-178	171-263	256-348	341-433	426-512	
BCG-HIV Gag (1)	3,500	800	540	>8,200	800	510	0
BCG-HIV Gag (2)	390	640	280	140	220	4,050	0
BCG-HIV Gag (3)	0	0	0	140	1,450	860	0
BCG	0	0	0	0	0	0	0
None	0	0	0	0	0	0	0

Spleen cells were obtained from three mice inoculated with BCG-HIV Gag recombinants, three mice injected with nonrecombinant BCG and three untreated mice; spleen cells from mice injected with nonrecombinant BCG were pooled, as were cells from the untreated mice. The cells (10^7 per ml) were stimulated with various peptides and γ -interferon levels (pg ml^{-1}) in cell supernatants were measured in a solid-phase enzyme-immunoassay (Genzyme) 4 days later²³, as described in the legend to Fig. 3. Supernatants were diluted 1:10 where necessary to obtain γ -interferon values within the linear range of the assay (260 – $4,100 \text{ pg ml}^{-1}$), values below 100 pg ml^{-1} were considered negative. Thirty overlapping HIV-1 Gag peptides (25 amino acids each and containing eight overlapping amino-acid residues) covering the entire Gag sequence²⁴ were grouped in six pools of five peptides each, and used at a concentration of $10 \mu\text{g ml}^{-1}$ per peptide for stimulation. Five unrelated peptides were pooled and used at similar concentrations as a negative control.

By comparing the signal obtained with known amounts of HIV-1 p24 with that obtained in lysates of BCG-HIV Gag recombinants, we estimated that the amount of full-length Gag precursor protein that accumulates in log-phase cells is $\sim 0.1\%$ of total BCG protein. Proteolytic fragments of the HIV-1 Gag, Pol and Env precursor proteins were also detected. Thus, the total amount of HIV polypeptides that accumulate in the BCG recombinants may be considerably higher than 0.1% of total protein.

When present on the high copy pYUB12 plasmid, the hsp70 promoter is used constitutively at high levels in the absence of heat shock, as the HIV-1 polypeptides did not accumulate to substantially higher levels after heat shock. In contrast, when integrated as a single copy in the BCG genome, lower levels of HIV-1 Gag are produced under the control of the hsp70 promoter at normal temperatures, and heat shock causes an increase in the accumulation of the foreign protein to levels similar to those found with the high-copy autonomous vector (data not shown).

We investigated the ability of BCG recombinants to induce

murine immune responses to the foreign proteins produced in BCG. BALB/c mice were inoculated with one primary dose of 5×10^6 wild-type BCG, 5×10^6 BCG-HIV Gag recombinants, or 5×10^6 BCG-HIV Env recombinant bacilli to study antibody responses. The mice were inoculated intradermally or intravenously. Mice were bled 5 weeks after inoculation and the sera were used in enzyme-linked immunosorbent assay (ELISA) and to probe western-blot strips containing HIV proteins (Fig. 2). Although none of the mice vaccinated with nonrecombinant BCG had detectable antibodies to Gag or Env, three mice out of five vaccinated with BCG-HIV Gag and one out of five vaccinated with BCG-HIV Env had detectable levels of IgG antibodies reactive against Gag (p24 and p17) and Env (gp160) proteins. All the mice producing antibodies against HIV-1 proteins had been inoculated with BCG recombinants intravenously. The levels of HIV-specific antibodies were generally low in the antibody-positive mice when tested by ELISA, and signals were detected when the sera were diluted no more than 1:50 in the western-blot assay. Nonetheless, these results

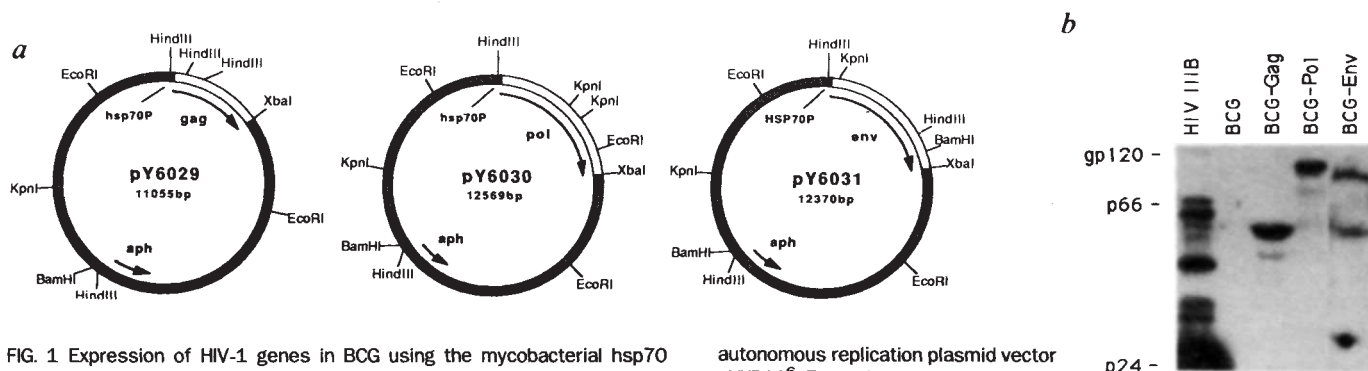


FIG. 1 Expression of HIV-1 genes in BCG using the mycobacterial hsp70 promoter and ribosome binding site. *a*, Three different plasmids containing HIV Gag, Pol or Env open reading frames were constructed. DNA fragments containing the coding sequence of HIV-1 Gag, Pol and Env were synthesized by polymerase chain reaction (PCR), using the plasmid pHXB2²⁵ as a template, and using oligonucleotide primers specific for each gene. The upstream primers contained an *Nco*I site that overlapped the AUG translation initiation codon of each HIV-1 gene, and the downstream primer contained an *Xba*I site immediately after the translation stop codon. *b*, Western blot of lysates of BCG recombinants probed with HIV-positive human serum. Lane 1: HIV-1 lysate containing 150 ng p24 protein; lane 2: lysate of wild-type *M. bovis* BCG; lanes 3, 4, 5: lysates of *M. bovis* BCG recombinants expressing HIV Gag, Pol and Env polypeptides, respectively.

METHODS. To attach the mycobacterial hsp70 promoter and ribosome binding site to each HIV-1 gene, a pUC18 plasmid containing a 1.8-kb segment of the *M. tuberculosis* hsp70 gene (pY6013) was digested with *Nco*I and *Xba*I to remove the hsp70 protein coding sequence (the hsp70 ATG translation initiation codon sequence overlaps an *Nco*I site), leaving the ATG and 155 bp of upstream hsp70 promoter sequence. The vector DNA containing the hsp70 promoter was gel-purified and ligated to the *Nco*I-*Xba*I PCR-derived HIV-1 DNA fragments. HIV-1 genes and their associated mycobacterial regulatory sequences were transferred to the mycobacterial

autonomous replication plasmid vector pYUB12⁶. *Eco*RI-*Xba*I fragments, blunt-ended at the *Eco*RI site, were gel-purified from the first set of plasmids and were inserted between the *Eco*RI and the *Xba*I sites of pYUB12 to produce pY6029 (containing HIV-1 Gag), pY6030 (containing HIV-1 Pol) and pY6031 (containing HIV-1 Env). All manipulations followed previously described procedures²⁶. Plasmids pY6029, pY6030 and pY6031 were introduced into the Pasteur strain of *M. bovis* BCG by electroporation and cells were plated on Middlebrook-7H9 agar (Difco) supplemented with albumin dextrose complement (Difco), 0.25% tween 20, and $20 \mu\text{g ml}^{-1}$ kanamycin sulphate. After growth for three weeks at 37°C (BCG cells have a doubling time of 24 h), individual colonies were picked and expanded in suspension cultures in Middlebrook-7H9 broth (Difco) to mid-log phase. Aliquots of cells (1.5 ml) were harvested by centrifugation and pelleted cells were resuspended in $50 \mu\text{l}$ Laemmli buffer²⁷, sonicated with three 20-s pulses using a microtip sonicator, and incubated at 100°C for 4 min. Debris was pelleted by microfuge centrifugation for 2 min and $15 \mu\text{l}$ of each lysate was electrophoresed on 10% SDS-polyacrylamide gels. The separated proteins were transferred from the gel to a nitrocellulose membrane and probed with human serum positive for HIV antibodies, followed by ^{125}I -labelled Protein A as described in ref. 28. The X-ray film was exposed for 20 h, except for lane 5, which was exposed for 60 h.

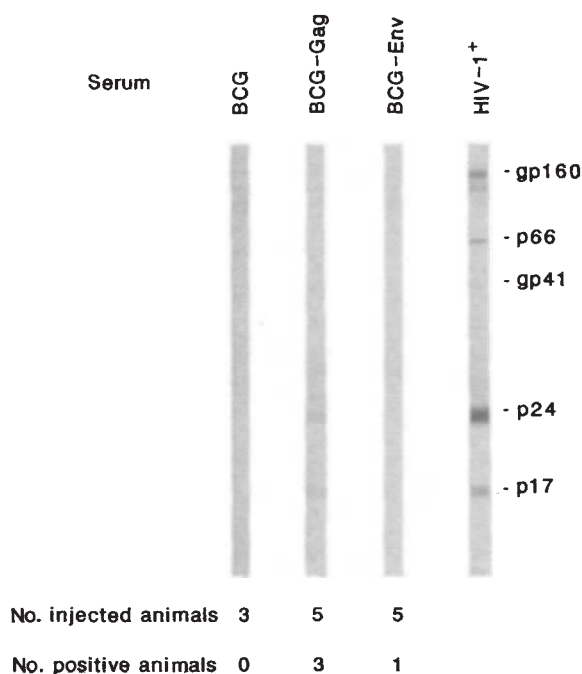


FIG. 2 Murine antibody response to vaccination with BCG recombinants expressing HIV-1 Gag and Env. Western-blot strips of HIV-1 proteins (DuPont) probed with serum from a mouse injected with nonrecombinant BCG (lane 1); with serum from a mouse injected with a BCG recombinant expressing HIV Gag (lane 2); with serum from a mouse injected with a BCG recombinant expressing HIV Env (lane 3). The blot was also probed with a human serum positive for HIV antibodies (lane 4). BALB/c mice were bled 5 weeks after injection with 5×10^6 cells of BCG or BCG recombinants.

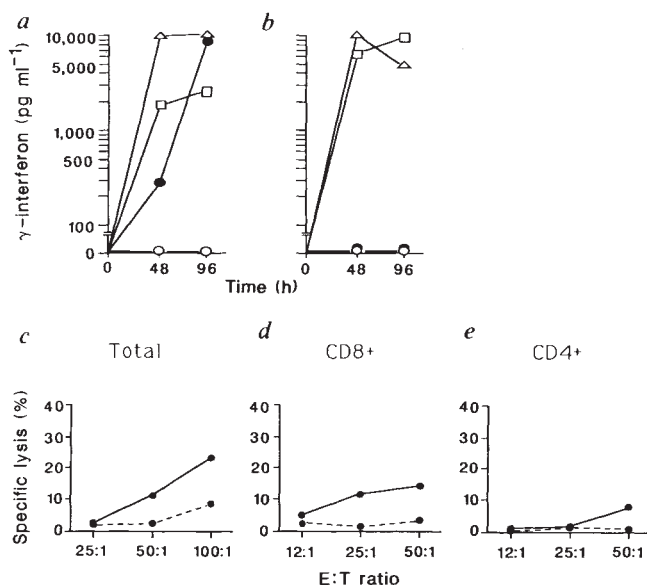


FIG. 3 Murine T-cell response to inoculation with BCG-HIV Gag recombinants. a, b, Levels of γ -interferon produced by spleen cells from mice inoculated with BCG-HIV Gag recombinants (a) or nonrecombinant BCG (b) after stimulation with $\square$, PPD ($50 \mu\text{g ml}^{-1}$); $\triangle$, conA ($5 \mu\text{g ml}^{-1}$); $\bullet$, HIV-Gag peptides ($10 \mu\text{g ml}^{-1}$ each); and $\circ$, control peptides ($10 \mu\text{g ml}^{-1}$ each). Levels of γ -interferon production were ascertained for three mice inoculated intravenously with BCG-HIV Gag recombinants and three mice inoculated intravenously with nonrecombinant BCG. The results shown in a and b were obtained from one mouse in each group; the other two mice in each group gave similar results. c, d, e, Specific cytotoxic activity of total, CD8+ and

demonstrate that BCG recombinants can elicit antibody responses to foreign proteins produced by the bacillus.

The ability of BCG recombinants to induce cellular immune responses to a foreign pathogen protein was investigated in mice inoculated with BCG-HIV Gag recombinants by measuring cytokine production and cytotoxic activity by spleen cells after stimulation with specific antigens. Cytotoxic T-lymphocytes (CTL), and to a lesser extent Th1 lymphocytes, produce γ -interferon when stimulated with specific antigens or with mitogens, and this response can be measured with clones or in bulk cultures^{14,15}. Mice inoculated intravenously with nonrecombinant BCG or with BCG-HIV Gag were boosted with 5×10^6 BCG or with 5×10^6 BCG-HIV Gag, respectively. Both HIV-1 p24 protein, which is a processed segment of the Gag polyprotein, and peptides covering the entire Gag amino-acid sequence were used as stimulating antigens in a γ -interferon production assay. Spleen cells from mice inoculated with BCG-HIV Gag recombinants produced substantial levels of γ -interferon when stimulated with HIV-1 Gag peptides, but not when exposed to similar levels of unrelated polypeptides (Fig. 3a). The level of γ -interferon produced in response to stimulation with HIV-1 Gag peptides was similar to that obtained when cells were stimulated with HIV-1 p24 (not shown) or with *M. tuberculosis* purified protein derivative (PPD) or concanavalin A (conA) (Fig. 3a). Spleen cells from mice inoculated with nonrecombinant BCG responded well to PPD and conA but were nonresponsive to the HIV Gag peptides (Fig. 3b). The spleen cell populations that produced γ -interferon in response to specific antigens also produced interleukin-2 (IL-2), as measured in a proliferation assay¹⁶ using the IL-2-dependent CTLL-2 cell line (not shown). Th1 lymphocytes are believed to be the major source of IL-2 in antigen-stimulated spleen cell populations^{14,15}. These results demonstrate that BCG recombinants can induce

CD4+ spleen cells from mice immunized with the BCG-HIV Gag recombinant (continuous line), and from mice injected with wild-type BCG (dashed line). METHODS. BALB/c mice initially injected with 5×10^6 nonrecombinant BCG or BCG-HIV Gag recombinants were boosted with a similar dose at 4 weeks and then at 8 weeks. Spleens were removed at week 9 and cells were cultured and tested for γ -interferon production as described in ref. 23. Spleen cells were stimulated at a concentration of 10^7 cells ml^{-1} with antigen or mitogen and supernatants were removed 48 h and 96 h later. Levels of γ -interferon in the supernatants were measured in duplicate with a solid-phase enzyme-immunoassay (Genzyme). Supernatants were diluted where necessary to obtain γ -interferon values within the linear range of the assay ($256\text{--}4,100 \text{ pg ml}^{-1}$); the background cutoff value was 100 pg ml^{-1} . A set of 30 overlapping HIV-1 Gag peptides covering the entire Gag sequence²⁴ was used for antigenic stimulation. Peptides were grouped in six pools of five peptides each, and used at a concentration of $10 \mu\text{g ml}^{-1}$ per peptide for stimulation. The results of stimulation with one of the six pools of HIV Gag peptides (representing Gag amino acids 256–348) or a pool containing five unrelated peptides as a negative control are shown in a and b. To investigate antigen-specific cytolytic activity in stimulated spleen cells from immunized mice, CD8+ and CD4+ T-cell populations were purified from total spleen cells by two rounds of negative selection (with complement and with either anti-CD4 or anti-CD8 antibodies) and characterized by two-colour FACS analysis (FITC-labelled antibody to CD8 (53-6.7, rat IgG2a, Becton Dickinson) and phycoerythrin-conjugated antibody to CD4 (GK1.5, rat IgG2b; Becton Dickinson)) as described in ref. 17. The FACS analysis demonstrated that the CD8+ and CD4+ T-cell populations were contaminated less than 1% by cells of the other phenotype. Target-cell lysis by total spleen or purified CD8+ and CD4+ T-cell was measured by the standard 4-h ^{51}Cr release assay¹⁸. Target cells were generated by incubating P815 tumour cells (American Type Culture Collection) with HIV Gag peptides at a concentration of $5 \mu\text{g ml}^{-1}$ per peptide and labelling with ^{51}Cr . In each assay, 10^4 target cells were incubated with varying numbers of effector cells. The percentage specific ^{51}Cr release was calculated from $100 \times (a-b)/(t-b)$, where a is ^{51}Cr release in the presence of effector cells, b is the spontaneous release from labelled target cells in the absence of effector cells, and t is the total ^{51}Cr content of the target cells (released by the addition of 1% Nonidet P-40).

murine cell-mediated immune responses to a foreign protein produced by the BCG recombinants, and are consistent with the involvement both of CTL and Th1 lymphocytes.

To investigate further the T-cell response to the BCG-HIV Gag recombinant, the antigen-specific cytolytic activity of spleen cells and of spleen cells depleted of either CD4 or CD8 cells was measured in a ^{51}Cr release assay^{17,18}. Spleen cells from mice immunized with the BCG-HIV Gag recombinant specifically lysed target cells pulsed with Gag peptides (Fig. 3c), as did spleen cells depleted of CD4⁺ cells (Fig. 3d). There was limited specific cytotoxicity with cells depleted of CD8⁺ cells (Fig. 3e); similarly low levels of specific cytotoxicity were observed when bulk spleen cells were preincubated with a monoclonal antibody that blocks CD8 function. These results indicate that most of the antigen-specific cytotoxic cells in the spleen population express CD8.

Multiple segments of HIV-1 Gag protein are immunogenic in mice inoculated with the BCG-HIV Gag recombinants (Table 1). Six pools of HIV-1 Gag peptides, each pool containing five overlapping 25 amino-acid peptides, were each used to stimulate spleen cells from mice inoculated with BCG-HIV Gag recombinants or nonrecombinant BCG. All six pools of Gag peptides stimulated substantial amounts of γ -interferon production, albeit to different levels, in spleen cells from mice injected intravenously with the BCG-HIV Gag recombinants. Spleen cells from mice inoculated with nonrecombinant BCG did not respond to any of the HIV-Gag peptides. Thus, the BCG-HIV Gag recombinants consistently induced T-cell responses to a variety of epitopes in the foreign protein.

The adjuvant properties of BCG and its cell wall components have previously been exploited in experimental vaccines in animals and in man. For example, mixtures of BCG and specific schistosomal antigens have been used successfully to protect mice in a model of schistosomiasis¹⁹. An adjuvant-antigen mixture of muramyl dipeptide and killed simian immunodeficiency virus (SIV) have provided partial protection against SIV infection in macaques^{20,21}; muramyl dipeptide is one of the components of mycobacterial cell walls that contributes to the adjuvant properties of BCG. Humans have been vaccinated with mixtures of BCG and killed *M. leprae* in large-scale trials to assess the efficacy of this leprosy vaccine candidate²².

Recombinant BCG vaccine vehicles can induce immune responses to foreign proteins produced by the bacillus, indicating that BCG can act simultaneously as an adjuvant and as a vehicle to produce and deliver selected antigens to the immune system. The ability to engineer BCG to produce one or more foreign pathogen antigens has several advantages over mixtures of mycobacterial adjuvant and pathogen antigens. Because the antigen continues to be produced by BCG replicating *in vivo*, a BCG recombinant may provide a more long-lived immune response to the pathogen of interest than that provided by the simple mixture of BCG and antigen. It may be more cost-effective to engineer BCG recombinants than to produce the mixture. Perhaps most importantly, the ease with which bacteria can be manipulated genetically makes it possible that features of the BCG vaccine vehicle can be tailored to maximize the desired immune responses. □

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Lower receptor avidity required for thymic clonal deletion than for effector T-cell function

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CLONAL deletion in the thymus plays a major part in T-cell tolerance to self antigens¹⁻³. But the mechanism of negative selection, its fine specificity and the threshold of affinity and avidity remains unknown. We have now examined these aspects of negative selection with mice expressing a transgenic T-cell receptor with specificity for lymphocytic choriomeningitis virus (LCMV) glycoprotein in association with the class I H-2D^b molecule. These mice were rendered tolerant to LCMV by neonatal infection with mutant LCMVs bearing point mutations in the T-cell epitope recognized by the transgenic T-cell receptor (ref. 4). Variant LCMVs were also tested for their ability to elicit antiviral responses in transgenic mice *in vivo* and *in vitro*. Comparison *in vivo* revealed that a low-avidity receptor interaction, which was unable to induce effector T cells in the periphery, was still sufficient for clonal deletion in the thymus.

Transgenic mice were generated using T-cell receptor (TCR) genes from the cytotoxic T-lymphocyte (CTL) clone P14, which specifically recognizes residues 32-42 of the LCMV glycoprotein in association with H-2D^b (refs 5-7). Mature CTL from these mice (H-2^b) efficiently lysed target cells (H-2^b) infected with wild-type LCMV virus, whereas there was about 10 times less lysis of targets infected with virus variant 8.1 and none with those infected with variant 8.7 (Fig. 1b, left). But these target cells were recognized equally by the CTL clone 50.1, which specifically recognizes residues 275-288 of the LCMV glycoprotein in association with H-2D^b. The glycoproteins of variants 8.1 and 8.7 contain an amino-acid substitution (Val 35 → Met for 8.1; Val 35 → Leu for 8.7) in the epitope recognized by P14 TCR (Fig. 1a). Peptides corresponding to this epitope (peptide 32-42) of wild-type and variant LCMV were similarly tested, and caused the same pattern of reactivity as that obtained with LCMV-infected target cells (Fig. 1b, middle). CTL specific for peptide 8.7, obtained from a spleen cell culture from C57BL/6 mice primed with variant 8.7, lysed all peptide-coated target cells equally well, indicating that the cells were properly coated with variant 8.1 and 8.7 peptides. CTL from transgenic mice

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could be activated *in vitro* by the wild-type peptide 32–42, but only partially by peptide 8.1 and not at all by peptide 8.7 (Fig. 1b, left). The ability of the P14 TCR to recognize the virus variants was further tested *in vivo*: P14 TCR transgenic mice efficiently cleared injected wild-type LCMV within 4 days, and eliminated virus variant 8.1 only at low dose infection and the variant 8.7 not at all (Fig. 1c). This result accords with the ability of CTL from transgenic mice primed with wild-type and variant LCMV to lyse the corresponding infected target cells (Fig. 1d). Together these data indicate that the P14 TCR is less reactive to cells infected with the virus variant 8.1, and that peripheral T cells bearing the transgenic P14 TCR are not activated either *in vitro* or *in vivo* by virus variant 8.7.

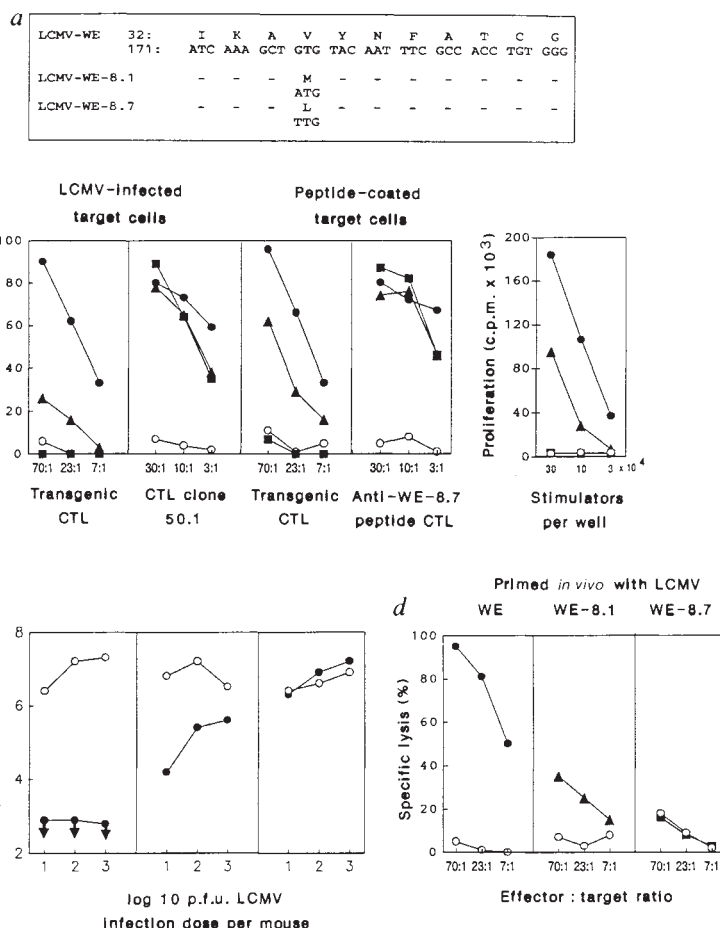
CTL specific for LCMV are clonally deleted in the thymus of P14 TCR transgenic mice neonatally tolerized with wild-type LCMV⁷. We thus examined the fate of T cells bearing P14 TCR in transgenic mice neonatally tolerized with the virus variants 8.1 or 8.7. The number of thymocytes in transgenic mice carrying variant 8.7 was comparable to that in uninfected transgenic mice ($0.8\text{--}1.4 \times 10^8$ per thymus), whereas thymus size and thymocyte numbers of transgenic mice carrying either wild-type or variant 8.1 virus were each reduced by about 5–10-fold. Thymocytes and lymph node cells from uninfected P14 TCR transgenic mice and transgenic mice carrying wild-type, 8.1 or 8.7 virus variants were stained with antibodies specific for CD3, CD4, CD8 and regions of the P14 TCR (V α 2 and V β 8) (Fig. 2). CD4⁺8⁺ and CD8⁺4⁺ thymocytes expressing intermediate and high amounts of P14 TCR were completely deleted in those mice carrying the wild-type or 8.1 variant virus, and decreased in number (~30–40%) in those carrying the 8.7 variant. The proportion of V α 2⁺CD8⁺/V β 8⁺CD8⁺ lymph node cells was 58% in uninfected

mice, 16–19% in mice carrying the 8.7 variant virus and 0.8–1% in those carrying the wild-type or the 8.1 variant virus. Among the peripheral CD8⁺ T-cell population, the proportion of cells expressing V α 2/V β 8 was reduced from 95–98% in the controls to 73–83% in 8.7-variant carriers, and to 16–23% in 8.1-variant or wild-type carriers. Thus, thymocytes expressing P14 TCR were deleted in 8.1-variant carriers as efficiently as in those carrying wild-type virus, and there was sufficient interaction between the P14 TCR and the 8.7 variant to delete a substantial proportion of thymocytes.

The tolerance of T cells to the virus in carriers was examined functionally. In a 3-day stimulation assay *in vitro*, lymph node and spleen cells from uninfected and 8.7-variant carrier mice, but not wild-type or 8.1-variant carriers, showed a primary immune response specific for LCMV by proliferation and cell-mediated lysis (Fig. 3). The proliferative response of lymph node cells from the 8.7-variant carriers was reduced by about threefold, whereas the lytic activity of the stimulated culture (~90% CD8⁺ cells) was comparable to that of the uninfected controls. The reduced proliferation of cells specific for the virus in 8.7-variant carriers reflected the fewer CD8⁺ lymph node cells expressing P14 TCR (Fig. 2b, middle), indicating that the transgenic TCR expressing CTL which were not deleted were not anergic^{8–10}.

To establish that the partial deletion of a thymocyte subset in 8.7-variant carriers was not the result of an unspecific inhibition of T-cell development by the virus infection, we examined H-2^d P14 transgenic mice carrying the 8.7 virus. Because H-2^d molecules cannot present peptide 32–42 to the P14 receptor, specific deletion was not expected; indeed, it did not occur in the thymus (Fig. 4) nor in the periphery (not shown). Thus, the

FIG. 1 Reactivity of CTL expressing P14 TCR against the wild-type LCMV-WE and its variants. **a**, Nucleotide and deduced amino-acid sequence (single-letter code) of the T-cell epitope (residues 32–42 of glycoprotein) of the wild-type virus and its 8.1 and 8.7 variants. **b**, *In vitro* reactivity of P14 TCR tested in a ⁵¹Cr-release assay (left) and in a cell proliferation assay (right) on uninfected (○) and on either wild-type (●), 8.1 (▲) or 8.7 (■) variant-infected or corresponding peptide-coated MC57G (H-2^b) target cells. Proliferation assays were done with uncoated (○) or peptide-coated (●, ▲, ■) C57BL/6 (H-2^b) stimulator spleen cells. **c**, *In vivo* reactivity of CTL expressing P14 TCR in mice, assayed by virus elimination. Transgenic (●) and transgene-negative littermates (○) were infected with the indicated dose of wild-type virus (left), 8.1 (middle) and 8.7 (right) variants and viral titres were determined in the spleen after 4 days. **d**, Activity of CTL in transgenic mice primed and tested with the indicated virus isolate. **METHODS**. **a**, Generation of the P14 $\alpha\beta$ TCR mice and the P14 T-cell epitope peptide 32–42 of wild-type virus and the 8.7 variant has been described previously^{4,7}. The epitope of the 8.1 variant was determined by complementary DNA synthesis, polymerase chain reaction amplification and DNA sequencing as described⁴. **b**, MC57G target cells (H-2^b) were infected with wild-type virus (from F. Lehmann-Grube, Hamburg), and its three-times plaque-purified 8.1 and 8.7 variants with a multiplicity of infection of 0.01–0.1 for 2 days. The peptides 32–42, IKAVYNFATCG, IKALYNFATCG and IKAMYNFATCG (single-letter amino-acid notation), (Neosystem Laboratoire, Strasbourg) were used at 100 μ M for coating M57G target cells. Effector cells were: T cells activated for 4 days *in vivo* with wild-type virus (10^6 plaque-forming units (p.f.u.)), CTL clone (50.1)¹⁵ specific for wild-type glycoprotein 275–288 and 8.7 peptide-specific CTL from a secondary *in vitro* MLC from variant 8.7-primed C57BL/6 mice. The lytic activity was determined in a 4–5 h ⁵¹Cr-release assay at the indicated effector:target ratio. Proliferation was measured by ³H-thymidine incorporation of 1.7×10^5 lymph node cells per well stimulated with the indicated number of irradiated, peptide-coated (200 μ M) C57BL/6 (H-2^b) spleen cells for 3 days. **c**, For the viral elimination assay, mice were infected by intravenous injection with the indicated dose of virus and viral titres were determined as described⁴. Titres are expressed in mean p.f.u. per g spleen on the basis



of the analysis of three mice per group, s.e.m. < 0.8 log 10. **d**, Mice were primed with 10^6 p.f.u. of the indicated viral isolate and the CTL activity was tested after 4 days on the indicated target cells.

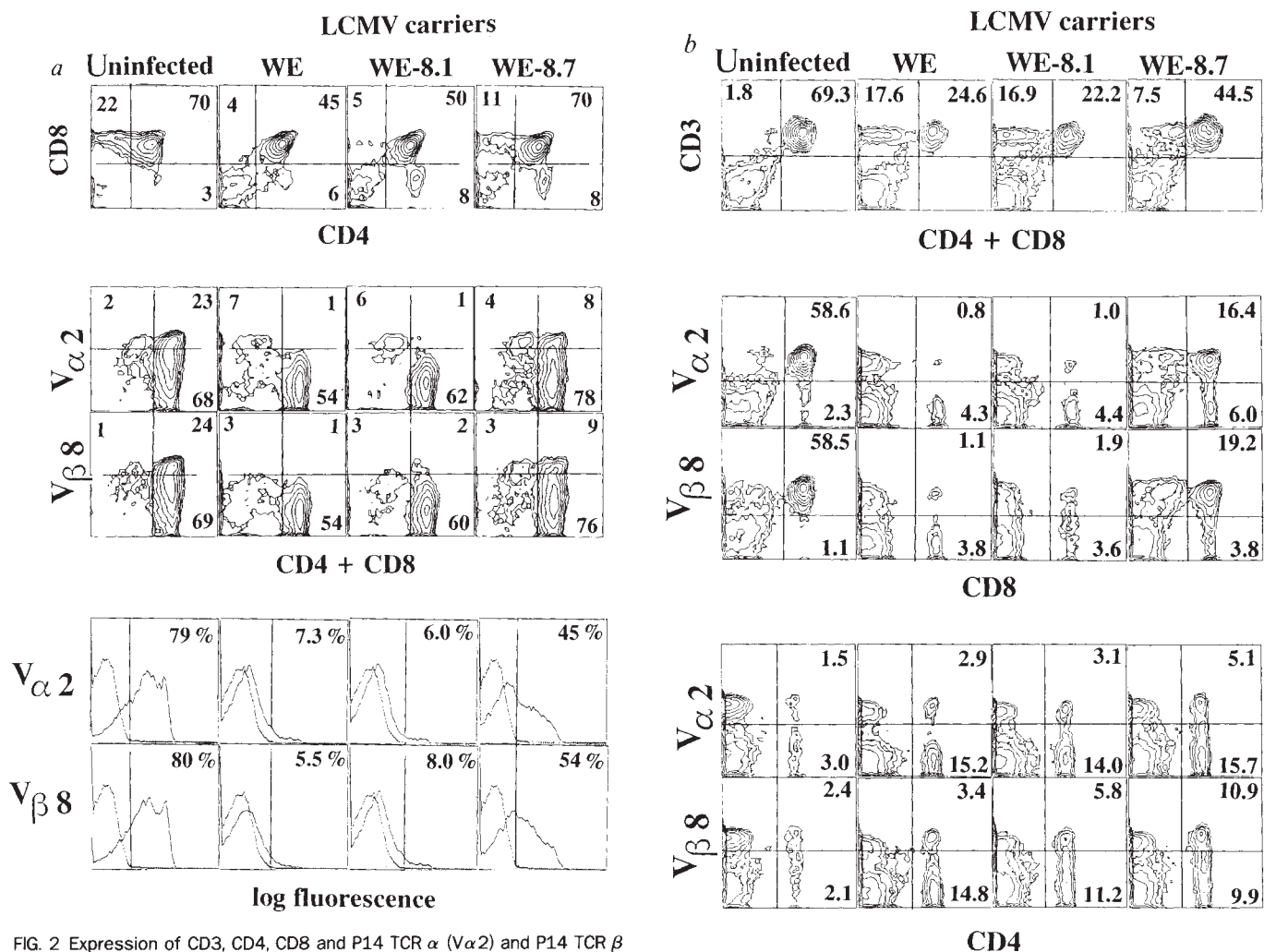


FIG. 2 Expression of CD3, CD4, CD8 and P14 TCR α ($V\alpha 2$) and P14 TCR β ($V\beta 8$) molecules on thymocytes and lymph node cells from uninfected transgenic mice and from transgenic mice carrying wild-type virus, 8.1 or 8.7 variants. **a**, Thymocytes were double-stained with antibodies specific for CD4 and CD8 (top) and CD4+CD8 and TCR $V\alpha 2$ - or TCR $V\beta 8$ - (middle). Single parameter histogram of TCR $V\alpha 2$ or $V\beta 8$ expression gated on $CD4^+8^+$, $CD4^+$ and $CD8^+$ thymocytes (bottom). **b**, Lymph node cells were double-stained with antibodies specific for CD4+CD8 and CD3 (top), CD8 and TCR $V\alpha 2$ or TCR $V\beta 8$ (middle) and CD4 and TCR $V\alpha 2$ or TCR $V\beta 8$ (bottom).

METHODS. Virus carriers were generated by neonatal injection (<24 h after birth, intraperitoneal injection of 5×10^6 p.f.u. per newborn mouse of wild-type LCMV-WE or its 8.1 or 8.7 variants). The presence of infectious virus

in the blood was tested for each mouse and mice were used at 6–12 weeks old. Single-cell suspension of fresh thymocytes and mesenteric lymph nodes were stained in buffered salt solution with 2% FCS and 0.2% sodium azide for 30 min at 4 °C. First-step reagents were anti-CD4-PE, CD8-FITC, CD8-Biotin (Becton Dickinson), hybridoma supernatant of KT3 (anti-CD3) (ref. 16), KJ16 (anti- $V\beta 8.1+8.2$) (ref. 17) and B20.1 (anti- $V\alpha 2$, B. Mallisen, manuscript in preparation). Second-step reagents used were FITC-conjugated goat anti-rat IgG and PE-streptavidin (TAGO, California). Viable cells gated by a combination of narrow-angle forward light-scatter and perpendicular scatter were analysed on an EPICS profile analyser (Coulter) with four logarithmic scales.

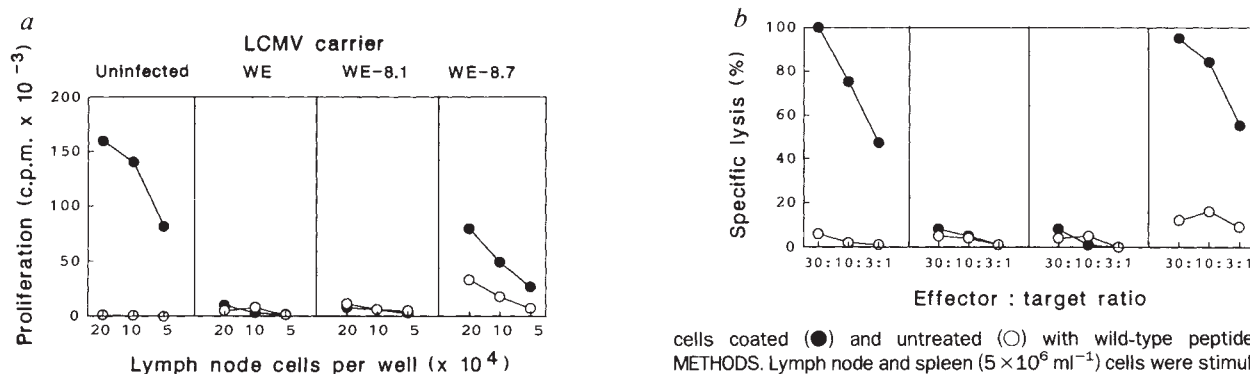


FIG. 3 Functional anti-LCMV-WE reactivity of P14 TCR carrier mice. Lymph node and spleen cells from uninfected and from carrier mice were cultured *in vitro* with uninfected (○) or with wild-type-infected peritoneal macrophages (●) and the proliferative response (**a**) and the lytic activity (**b**) of the virus-stimulated cultures against MC57G target

cells coated (●) and untreated (○) with wild-type peptide 32–42. METHODS. Lymph node and spleen (5×10^6 ml $^{-1}$) cells were stimulated with irradiated (3,000 rad) wild-type-infected peritoneal macrophages (4×10^5 ml $^{-1}$) as described⁵. Cell proliferation was determined after 3 days by 3 H-labelled thymidine uptake. After 3 days, the lytic activity of the spleen cell cultures were tested on MC57G(H-2^b) target cells coated with wild-type peptide 32–42 (IKAVYNFATCG) (100 μ M) in a 4–5 h 51 Cr-release assay at the indicated recovered effector to target cell ratio.

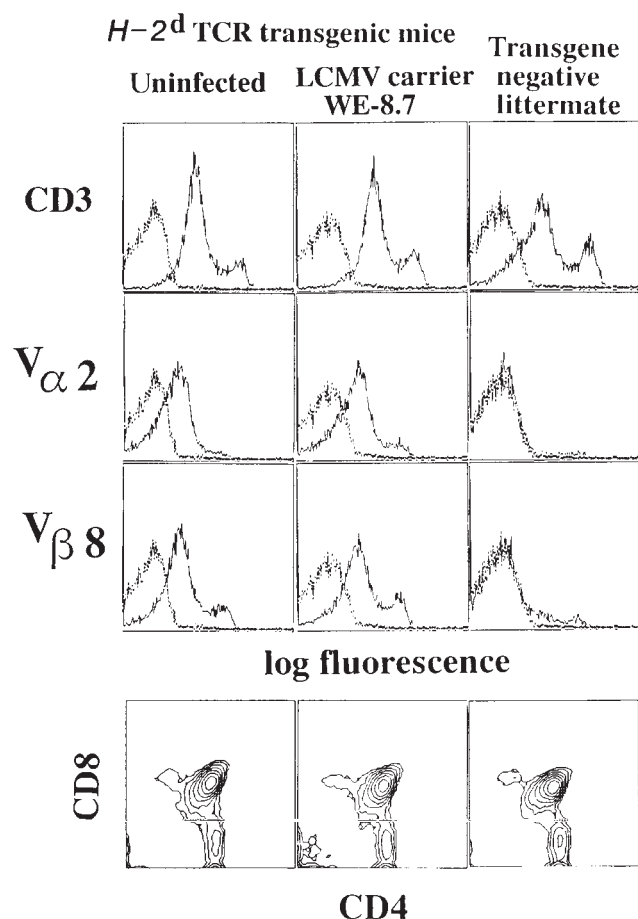


FIG. 4 Virus infection does not interfere with thymocytes development in P14 TCR-H-2^d transgenic mice carrying 8.7 variant virus. Thymocytes were single-stained with CD3, TCR V α 2 and TCR V β 8 and double-stained with CD4- and CD8-specific antibodies. Thymocytes from H-2^d mice express the P14 TCR at low amounts due to the lack of positive selection when compared with H-2^b LCMV carrier mice which clonally delete thymocytes even at this stage¹⁸.

METHODS. H-2^d animals have been derived from matings with BALB/c mice. Conditions for staining and the generations of LCMV carrier mice are as described in Fig. 2.

partial deletion in H-2^b 8.7-variant carriers was due to a specific TCR-antigen interaction.

This study demonstrates that in mice transgenic for a TCR specific for an LCMV peptide plus D^b, the TCR-ligand interaction can activate peripheral T cells poorly (for a variant virus 8.1) or not at all (for variant 8.7). Nevertheless the interaction causes complete (variant 8.1) or partial (8.7 variant) clonal deletion of transgene-bearing thymocytes in the thymus. This accords with the high sensitivity of staphylococcal enterotoxin B-induced clonal deletion *in vitro*¹¹. The deletion in 8.7-variant carriers occurred earlier in thymocyte development than that induced by Mls-1^a examined in the same TCR transgenic model (ref. 7). This is because thymocytes expressing both intermediate and high numbers of P14 TCR were reduced in number. This could argue against the possibility that it is primarily the avidity of the receptor that determines the developmental stage at which deletion occurs, and indicates that the quality of the antigen itself influences negative selection. These data also provide an explanation for why T cells expressing TCR V β 3, 5, 9 or 11 are deleted in Mls-3^a, Mls-1^a or I-E⁺ mice, whereas most mature T cells expressing these chains are nonreactive to the deleting antigen *in vitro*¹²⁻¹⁴ and also certain TCR V β -deletion elements do not (or only weakly) stimulate a primary mixed-lymphocyte reaction. The presence of functional CTL carrying P14 TCR in 8.7-variant carrier mice and in similar mice expressing Mls-1^a (ref. 7) suggests that deletion in the thymus could be 'leaky' if

the receptor interaction is of low avidity. Nevertheless, the 'margin for safety' in the clonal deletion process probably prevents induction of mature effector T cells for thymic self antigens. □

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A novel class II MHC molecule with unusual tissue distribution

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THE repertoire of mature class II-restricted T cells is generated through a complex process of selection whereby early T cells confront class II molecules in the thymus, especially on epithelial cells¹⁻³. Expression of class II molecules on such cells is prominent both in the cortex and in the medulla^{4,5}. We have identified a novel class II molecule, H-2O, which is expressed only in epithelial cells of the thymic medulla and in B cells. The unusual tissue distribution and the nonpolymorphic nature of H-2O suggest that its function is different from that of classical class II molecules.

As no isotypic α chain has been detected, the class II $\text{A}\beta$ 2 gene (now known as Ob) is probably a pseudogene, although transcripts have been identified^{6,7}. The very low amount of correctly spliced messenger RNA and its lack of regulation by interferon- γ (ref. 7) support its pseudogene status. To determine whether the Ob gene is translated, we made a rabbit peptide antiserum against the cytoplasmic tail sequence⁸. The peptide antiserum was used in biosynthetic pulse-chase experiments with B10.M (H-2^f) spleen cells. This strain of mice was chosen because it does not manufacture H-2E α or H-2E β chains^{9,10}. Immunoprecipitated molecules were subjected to two-dimensional electrophoretic analyses with and without treatment with endoglycosidase H (endo H). The antiserum precipitated material that separated into three major spots (Fig. 1Aa). The most basic spot corresponded to the invariant chain (Ii) (see below). Treatment with endo H reduced the relative molecular mass (M_r) of Ii and the two H-2O polypeptides (Fig. 1Ab). The material in the middle spot (denoted O α) probably contained two N-linked high-mannose carbohydrates, as its endo H-induced shift was similar to that for Ii. Accordingly, the acidic O β chain (see below) must contain only one carbohydrate moiety as its shift was smaller. This interpretation is consistent with the O β amino-acid sequence, which contains only one recognition site for N-linked carbohydrates⁶.

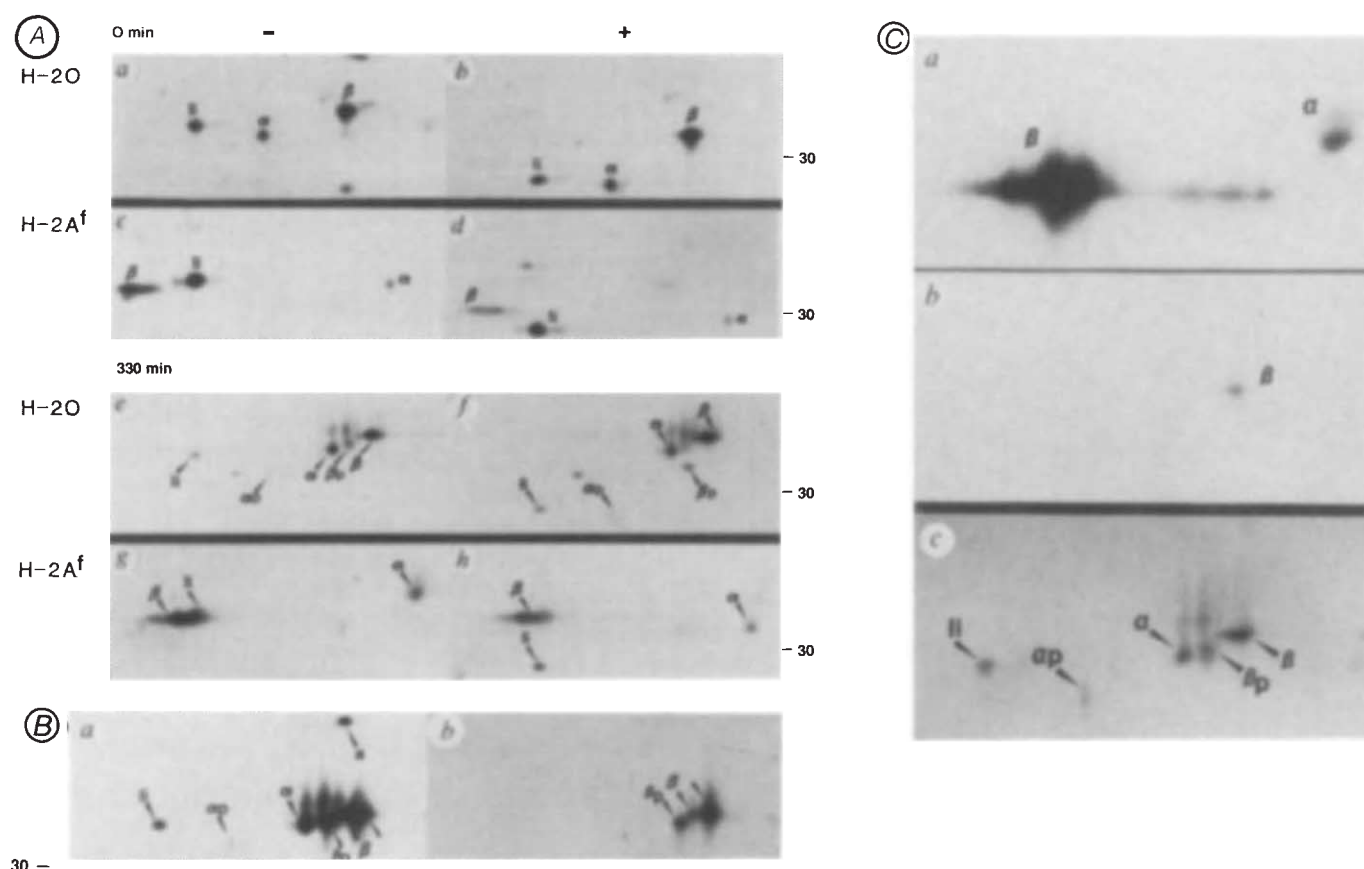


FIG. 1 The H-20 β chain is associated with a novel, nonpolymorphic α chain. **A**, B10.M splenocytes were pulse-labelled for 20 min and analysed immediately (0 min) or after a chase period (330 min). Class II molecules were immunoprecipitated with K507 (anti-H-20 β) (a, b, e, f) and K501 (anti-H-2A β) (b, d, g, h) and subjected to two-dimensional gel electrophoresis without (a, c, e, g) or with (b, d, f, h) endo H treatment. **B**, To identify which spots corresponded to the H-20 α and H-20 β chains, B10.M splenocytes, labelled for 4 h, were lysed and immunoprecipitated with K507. Part of the immunoprecipitate was analysed by two-dimensional gel electrophoresis (a), and the remaining precipitate was dissolved in SDS, diluted and reprecipitated with K507 before the electrophoretic analysis (b). **C**, Cell-surface expression of molecules was examined by labelling DBA/1 splenocytes by lactoperoxidase-catalysed iodination. For reasons of comparison, H-2A molecules (a) and H-20 molecules (b) were immunoprecipitated with the anti-H-2A β serum (K501) and the anti-H-20 β serum (K507), respectively, and analysed by two-dimensional gel electrophoresis. To demonstrate that the iodinated H-20 β chain exclusively corresponded to terminally glycosylated chains, DBA/1 splenocytes were metabolically labelled for 1 h and chased for 2 h before immunoprecipitation with the anti-H-20 β serum and two-dimensional gel electrophoresis (c); α p and β p denote immature precursor forms, which do not contain processed carbohydrate moieties.

METHODS. Rabbit antisera were raised against KLH-conjugated peptides (RAQKGYYRTQSGNEASRESLHSQP for K507 (anti-O β) and RHRSQKGRGPP-PAGLLQ (single-letter amino-acid notation) for K501 (anti-A β)). The specific reactivities of the antisera were tested in ELISA assays using tail peptides from H-2E α , H-2E β and H-2A α as well as the ones used for immunization. There was no cross-reactivity. In immunoprecipitations from mouse fibroblasts transfected with cDNA coding for H-20 β or H-2A β , K507 only reacts with H-20 β and K501 only with H-2A β . Pretreatment of the antisera with the peptide used for immunization abolishes this reactivity, whereas pretreatment with an irrelevant tail peptide has no effect. Cell labelling, lysis, immunoprecipitation and first gel dimension nonequilibrium pH gradient electrophoresis gels (using ampholines pH 3.5–10) were done as described by Jones¹⁹, except that both [³⁵S]methionine and [³⁵S]cysteine were used. The H-20 complex in **B** was split by boiling for 5 min in 1% SDS in the absence of reducing agents, then the detergent was diluted 20 times in 1% Triton X-100 and H-20 β was reprecipitated. Endo H treatment and two-dimensional gels have been described in ref. 20. Surface iodinations using the lactoperoxidase-glucose oxidase method were carried out as described elsewhere²¹. That no labelled actin was precipitated from the iodinated cells confirmed that only surface proteins had been labelled. Cell viability was >95% based on trypan blue exclusion.

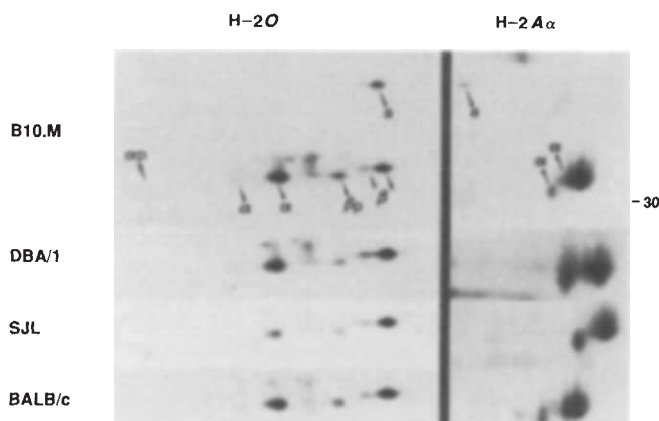


FIG. 2 Two-dimensional gel electrophoresis of H-20 and H-2A chains of different H-2 haplotypes. Splenocytes of the indicated mouse strains were metabolically labelled for 4 h. H-20 and H-2A molecules were immunoprecipitated with K507 and K501, respectively, and separated by electrophoresis. Only the H-2A α chain regions of the gels are shown. a, actin; α p and β p, the precursors of the H-20 chains.

METHODS. All procedures were as in Fig. 1. Two-dimensional gel electrophoretic separations were done as described¹⁹ using isoelectric focusing (ampholines pH 5–7 for H-20 precipitates, and pH 3.5–5 for H-2A precipitates) for the first dimension.

Identical experiments were done with a peptide antiserum against H-2A (Fig. 1Ac, d). The H-2A α , H-2A β and Ii spots were in the expected positions and were sensitive to endo H treatment. The only spot coinciding with the H-2O spots was Ii.

After 5.5 h of chase, most of the H-2O chains had become more acidic, which was most pronounced for the α chain (Fig.

1Ae). The charge changes and the resistance to endo H (Fig. 1Af) are consistent with the acquisition of sialic acid by both chains. Small amounts of material also occupied the same positions as the pulse-labelled spots (compare with Fig. 1Ae, and f, O α p and O β p). The chase period induced the expected changes in the A chains (Fig. 1Ag, h), but none of the H-2A chains was

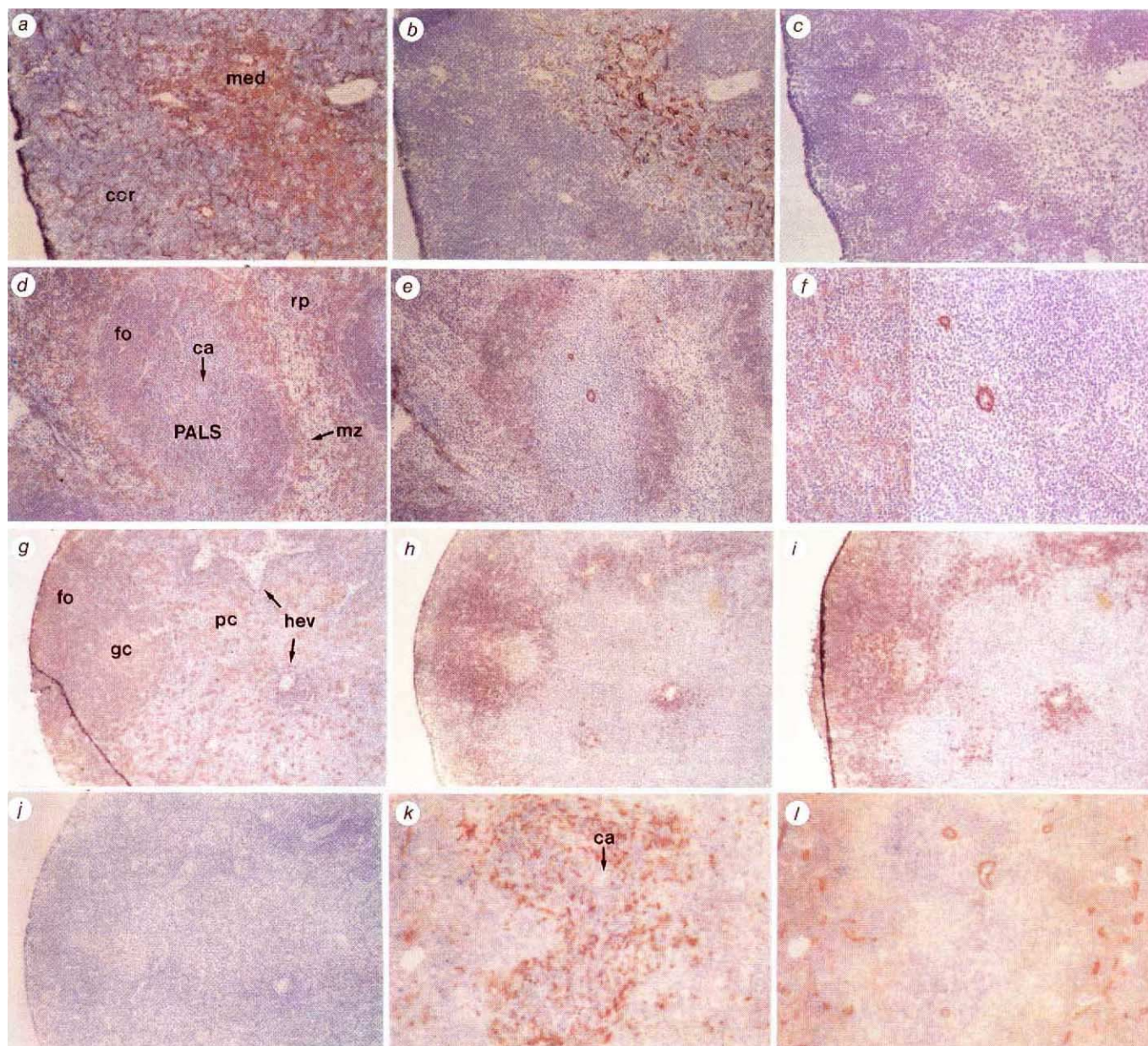


FIG. 3 Staining of lymphoid tissues by anti-H-2A and anti-H-2O β antibodies. *a-c*, In the thymus, H-2A expression (*a*) is uniform in the medulla (*med*), the site of marrow-derived Ia-positive antigen-presenting cells, and is also prominent in the network of epithelial cells in the cortex (*cor*); H-2O β expression (*b*) is not evident in the cortex and, in the medulla, is limited to stellate cells with features of epithelial cells; staining is abolished when the anti-H-2O β antibody is preincubated with the immunizing peptide (*c*). *d-f*, In spleen, H-2A expression (*d*) is evident in the follicles (*fo*) of the white pulp (on B cells), the periarteriolar lymphocyte sheaths (PALS) (on interdigitating dendritic cells), the marginal zone (*mz*) (on macrophages and B cells), and the red pulp (*rp*) (on various cell types). H-2O β expression (*e*) is limited to the follicles and central arterioles (*ca*) of the white pulp. High-power views of the PALS of the spleen (*f*) show H-2A expression on dendritic cells (*f*, left), H-2O β expression on arterioles but not on dendritic cells (*f*, centre) and no H-2O β staining after absorption with the immunizing peptide (*f*, right). *g-j*, In lymph nodes, H-2A expression (*g*) is prominent on the B cells of the follicles (*fo*) and on the scattered dendritic cells of the

paracortex (*pc*); germinal centres (*gc*) show only low H-2A expression; H-2O β expression (*h*) is limited to the follicles and to cells (presumably B cells) accumulated around the high endothelial venules (*hev*) of the paracortex; H-2O β expression closely resembles IgM μ expression (*i*); no H-2O β expression is seen after absorption with the immunizing peptide (*j*). In the collapsed spleen of irradiated mice, which contains dendritic cells but very few B cells, there is strong H-2A expression (*k*), whereas H-2O β is expressed only on central arterioles (*l*).

METHODS. Using BALB/c (H-2^d) mice, freshly cut frozen sections (5 μ m) fixed in acetone were incubated for 1 h with optimal concentrations of biotinylated anti-H-2A^d monoclonal antibody BP107 (ref. 22), anti-H-2O β antibody, or anti- μ antibody²³ and then incubated with horseradish peroxidase-conjugated streptavidin followed by the substrate 3-amino-9-ethylcarbazole. Sections were then counterstained with haematoxylin. To control for the specificity of staining, the anti-H-2O β antibody was absorbed with the antigenic H-2O β peptide for 1 h at 4 °C and airfuged for 30 min. Irradiated spleens were taken from BALB/c mice exposed to 1,000 rad 2 days before.

TABLE 1 Ob cDNA displays limited polymorphism

Strain	Haplotype	Base changes		Amino-acid changes	
C57Bl/6	<i>b</i>	—	—	—	—
SJL	<i>s</i>	—	—	—	—
B10.M	<i>f</i>	215	G → A	46	Met → Ile
CBA/J	<i>k</i>	287	G → A	—	—
		383	T → C	—	—
		446	C → T	—	—
DBA/1	<i>q</i>	446	C → T	—	—
		608	C → T	—	—
BALB/c	<i>d</i>	328	A → G	84	Lys → Arg
		383	T → C	—	—
		398	G → A	—	—

First-strand splenocyte cDNA was amplified by PCR with oligonucleotides specific for H-2Ob according to a published protocol¹⁷. The PCR products were cloned and exons two and three were sequenced¹⁸. At least two sequences from independent reverse transcriptions and PCR amplification were analysed from each haplotype.

coincident with the H-2O chains.

To find out which one of the H-2O chains corresponded to H-2Oβ, we carried out sequential immunoprecipitations with the H-2Oβ antiserum after solubilization of the first immunoprecipitate in SDS. The two-dimensional gel pattern confirmed that the more acidic, high-*M_r* component corresponded to the H-2Oβ chain (Fig. 1B).

To examine whether H-2O molecules are expressed at the cell surface, DBA/1 splenocytes were subjected to lactoperoxidase-catalysed iodination. Analyses of immunoprecipitated H-2A and H-2O molecules by two-dimensional gel electrophoresis confirmed that class II α chains are poorly iodinated^{11,12} (Fig. 1Ba, b). That the iodinated H-2Oβ chains had electrophoretic properties indistinguishable from those of metabolically labelled, terminally glycosylated H-2Oβ chains (Fig. 1Ab, c) suggests that H-2O molecules are expressed at the cell surface.

The electrophoretic analyses demonstrated that the H-2Oβ chain pairs with a novel α chain and that the heterodimer associates with Ii. H-2O is transported out of the endoplasmic reticulum, divests itself of Ii at late stages of the intracellular transport and is expressed at the cell surface. Like H-2A and H-2E α chains, the H-2Oα chain contains two *N*-linked carbohydrate units, but in contrast to the conventional class II α chains¹³, both moieties become resistant to digestion by endo H. The unusually rapid electrophoretic migration of the H-2Oα chain in comparison to other class II α chains is presently unexplained. Cloning of the H-2Oα complementary DNA may resolve this issue.

Limited restriction-fragment-length polymorphism analyses suggested that the Ob gene is much less polymorphic than the Ab and Eb genes⁶. To examine this further, we amplified by polymerase chain reaction (PCR) the Oβ cDNAs comprising six different H-2 haplotypes. The sequences corresponding to the second and third exons of the Ob gene were determined. Only two replacement substitutions were observed (Table 1), both being conservative. Neither position 46 nor position 84 are believed to contribute to the peptide-binding groove of class II molecules¹⁴. We also examined potential H-2O polymorphism by two-dimensional electrophoresis. Figure 2 summarizes the results for four strains of mice with different H-2 haplotypes. To ascertain that the technique adopted resolved allelic forms of H-2α chains, we analysed H-2A molecules from the four mouse strains in the same way; each allelic Aα chain gave a distinct pattern. By contrast, the H-2Oβ chains displayed the same apparent pI, regardless of the mouse strain from which they were derived, which confirms the low degree of H-2Oβ polymorphism. Thus, appropriate amounts of immunoprecipitates from the various mouse strains were mixed pairwise before

two-dimensional gel electrophoresis. As the same number of spots were encountered for the mixed and single samples, it can be concluded that no haplotype-specific differences were observed. Also the H-2Oα chain displayed no measurable polymorphism.

To examine the tissue distribution of the H-2Oβ, we stained frozen sections of various organs with the peptide antibodies and, as a control, with antibodies against H-2A molecules. In thymus H-2A staining was present throughout the medulla and on cortical epithelial cells. H-2Oβ staining, by contrast, was limited to aggregates of stellate cells in the medulla. These cells appeared to be epithelial cells because an identical pattern of staining was observed with an antibody specific for a subset of medullary epithelial cells¹⁵. In spleen and lymph nodes, H-2A staining was dense in the B-dependent areas (follicles in peripheral region of spleen white pulp and superficial cortex of lymph nodes) and also in the sites occupied by macrophages (marginal zone of spleen) and dendritic cells (periarteriolar lymphocyte sheath of spleen and paracortex of lymph nodes). H-2Oβ staining, however, was limited to the B-dependent areas of spleen and lymph nodes, and closely resembled the staining pattern for immunoglobulin μ chains. The collapsed spleen of recently irradiated mice, which is enriched in macrophages and dendritic cells but almost devoid of B cells, showed strong H-2A staining but very limited H-2Oβ staining. The anti-H-2Oβ reagent also produced strong staining of blood vessels, especially the central arterioles of the spleen white pulp. H-2Oβ staining of B cells and blood vessels was specific because preincubation with the antigenic peptide abolished staining of both cell types.

These data demonstrate that the H-2Oβ chain has a more limited tissue distribution than H-2A molecules. In thymus the H-2Oβ chain is absent from the cortex, suggesting that it might not be involved in the positive selection of T cells bearing αβ-receptors. But the occurrence of H-2Oβ chains on a subset of medullary epithelial cells suggests that H-2O molecules might be involved in negative selection. In the periphery, the H-2Oβ chain expression seems confined to B-lymphocytes^{7,16}. The apparent lack of H-2O molecules on macrophages and dendritic cells, which are regarded as essential antigen-presenting cells, reinforces the notion that H-2O molecules have a function different from the classical class II molecules. In view of the structural similarity between H-2O and H-2A or E molecules, it would not be surprising if H-2O molecules were recognized by T-cell receptors. The nonpolymorphic nature of H-2O molecules and the limited germ-line repertoire of T cell receptor γ and δ genes are reasons to investigate whether these two types of molecules might interact. □

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Rhodopsin-regulated calcium currents in *Chlamydomonas*

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THE unicellular alga *Chlamydomonas reinhardtii* responds to weak flashes of light by changing its swimming direction and to brighter flashes with transient backward swimming (the stop or phobic response)^{1,2}. In continuous light the cells swim towards or away from the light source (phototaxis)^{3,4}. This behaviour is controlled by a visual system with a rhodopsin as the functional photoreceptor^{5,6}. Physiological experiments under different ionic conditions have suggested that ionic processes are involved in the signal transduction from the photoreceptor to the flagella^{1,2,4}. Here we show by ion current measurements that there are two distinct light-regulated inward currents which are localized in the eyespot and in the flagellar region of the cell. From the kinetics and the rhodopsin action spectrum of these photocurrents we conclude that they are part of the rhodopsin-regulated signal transduction chain controlling the cellular behaviour in light. Both photocurrents are Ca^{2+} -dependent and are suppressed by the Ca^{2+} -channel inhibitors verapamil and pimozone, suggesting that the photoreceptor current and probably the flagellar current are both carried by Ca^{2+} .

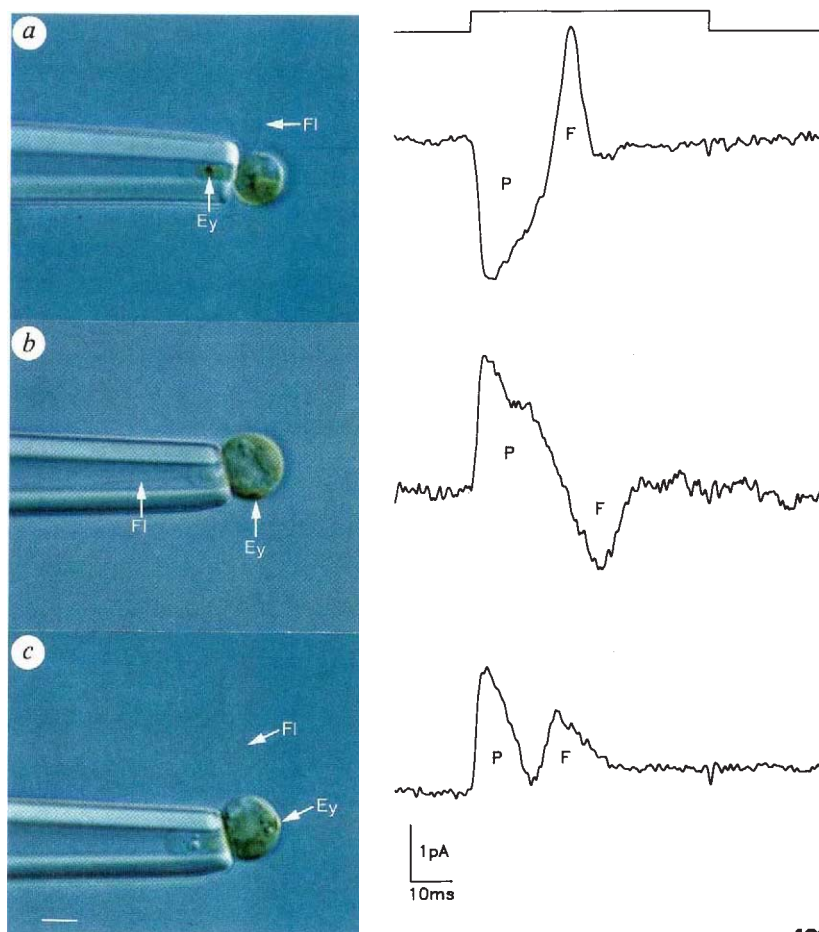
The green alga *Chlamydomonas* responds to bright flashes of green light by means of two distinct ion current components which we have recorded in cell wall-deficient mutants using the suction pipette technique^{7,8} (Fig. 1). The photocurrents are transient and occur in a time range of 0.5 to 50 ms after flash or onset of bright light, fast enough to trigger movement

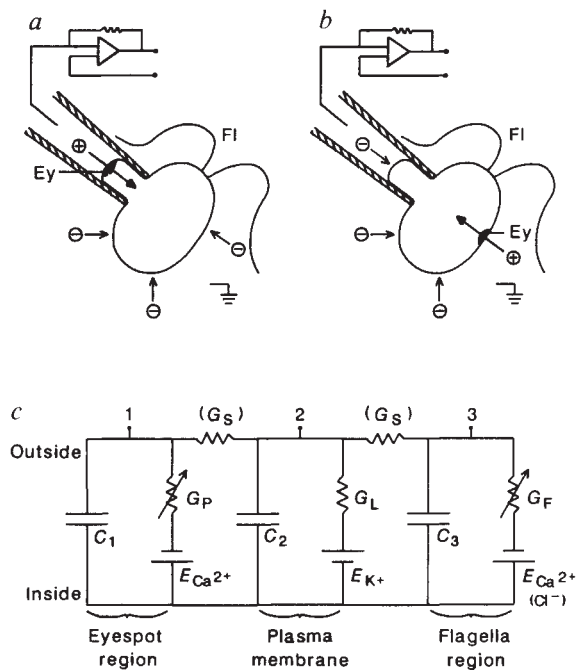
responses. The first current originates in the eyespot area (photoreceptor current) and the second in the region of the flagella (flagellar current). When the source region of the respective current is in the pipette, a downward current signal is recorded which corresponds to a cation influx (Fig. 1a, b). When eyespot or flagella are outside the pipette, the signals have the opposite sign. As delay and kinetics of the corresponding signals are identical, it is very unlikely that the positive signals reflect secondary processes as calcium- or voltage-activated currents in the plasma membrane. Instead, upward currents presumably reflect capacitive displacement currents, which are a direct consequence of the localized inward currents in the eyespot or the flagellar region. This is explained in more detail in Fig. 2. Most experiments reported below were carried out in the 'eyespot-out, flagella-out' configuration.

The photoreceptor current (first component) is graded with photon exposure (Fig. 3a), exhibiting a sigmoidal stimulus-response curve of the peak current (Fig. 3b). Its dynamic range (between 17% and 90% of the maximal amplitude) extends over 1.5 decades of photon exposure. The photoreceptor current occurs with virtually no delay (less than 500 μs) after the flash and decays faster at high photon exposure. The light sensitivity of the cell depends on the orientation of the eyespot relative to the actinic light. When the eyespot points away from the light source, the stimulus-response curve is shifted parallel by a factor of about eight to higher photon exposure, compared with a situation where it faces the light source directly (data not shown). This shift is a measure for the effective front-to-back contrast of the cell at the site of the photoreceptor. It causes the cyclic modulation of the received light signal during the helical swimming path, enabling the cell to align its swimming direction with the light incidence⁹. Photoreceptor currents show a rhodopsin action spectrum that peaks at 494 nm (Fig. 3c), which is similar

FIG. 1 Light-induced photoreceptor (P) and flagellar (F) currents, whose sign depends on the orientation of the cell in the pipette. **a**, The eyespot (Ey) is inside the pipette; **b**, the flagella (FI) are inside the pipette; and **c**, eyespot and flagella are outside the pipette. Scale bar, 5 μm .

METHODS. Cell wall-defective *C. reinhardtii* strain CW2 cells were grown and differentiated into gametes overnight²¹. Phototactically active cells were photoselected 3 times² in 5 mM HEPES, 10 mM Cl^- , 1 mM K^+ , 300 μM Ca^{2+} , 200 μM 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)²², adjusted with *N*-methyl-D-glucamine to pH 6.8. After dark adaptation for 1–3 h, the cells were sucked by low-pressure application into the pipette (3 μm tip; 100 M Ω ; filled with the same buffer) until the resistance reached 200–250 M Ω . All manipulations were under microscopic control with infrared light (RG 830 filter; Schott, Mainz; 250 W m^{-2}). Cells were stimulated with light pulses (6×10^{20} photons $\text{m}^{-2} \text{s}^{-1}$; λ_{max} 495 nm; 16-nm half-band width; single-cavity interference filter; Schott, Mainz) of 60-ms duration (top trace). Currents were recorded under voltage clamp (0 mV between bath and pipette) with a patch clamp amplifier (EPC-7; List, Darmstadt) and filtered with a 3-kHz low-pass Bessel filter. Data were stored and processed with the help of a labmaster DMA board and pClamp 5.5 software (both from Axon Instruments, Foster City). Current records were filtered with a digital gaussian filter to 500 Hz and averaged 50 times (a), 20 times (b), or 10 times (c). The temperature was maintained at $19 \pm 1^\circ\text{C}$. Cells were photographed using Nomarsky interference optics.





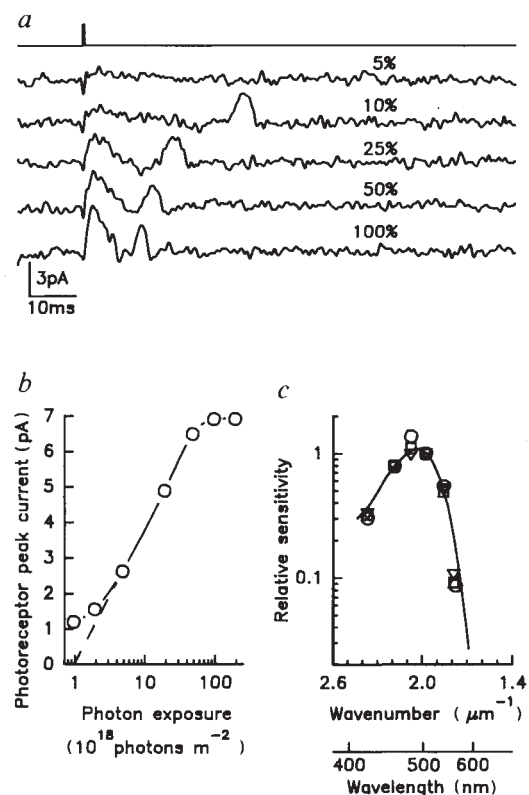
to the action spectra for phototaxis⁵ (503 nm) and flash-induced stop responses¹⁰ (495 nm). It also matches the *in vitro* absorption of *Chlamydomonas* rhodopsin in purified membranes⁶ (495 nm), suggesting that the observed photocurrents are rhodopsin-mediated processes.

The flagellar current appears only at photon exposures above a certain threshold and, other than the photoreceptor current, its shape and amplitude are unchanged over a wide exposure range (Fig. 3a). Like an action potential, it is an all-or-none effect, and in this respect it resembles the physiological stop

FIG. 3 a, Light titration of the electrical responses. The signals in a represent single events without averaging. They were recorded in the 'eyespot-out' configuration and with the eyespot facing the actinic light (0.3-ms duration; λ_{max} 495 nm; 16-nm bandwidth; 100% corresponds to 2×10^{19} photons m^{-2}). b, Plot of the photoreceptor peak current versus photon exposure on a log scale. Each data point represents an average of 15 recordings. c, Threshold action spectrum of the photoreceptor peak current. Stimulus-response curves were recorded from three different cells ($\square$, $\circ$, ∇) with the eyespot always facing the actinic light. Threshold values were obtained by extrapolating the linear part (containing at least 3 data-points) down to zero response, as indicated by the dashed line in b. Each datapoint represents the mean of 10 recordings. The absolute light sensitivities (1/threshold photon exposure) at 505 nm were 1.3×10^{-18} , 1.3×10^{-18} , and 5×10^{-19} photons⁻¹ m^2 . They were normalized to a value of 1 at 505 nm. The relative sensitivities were plotted on a log scale versus wavenumber. The solid line is Dartnall's standard curve for a rhodopsin absorption²³. Light of the appropriate wavelength was selected by single cavity interference filters with 5–12 nm band width.

FIG. 2 a and b, Schematic representation of two measuring configurations, with eyespot inside (a) or outside (b) the pipette. Electrical processes in the flagellar region (FI) have been omitted for clarity. Photoexcitation leads to a transient increase in Ca^{2+} conductivity in the eyespot region (Ey). When the eyespot is in the pipette (a), Ca^{2+} flows from the pipette interior into the cell ($\oplus \rightarrow$), leading to a downward current signal. In most cases—one is seen in Fig. 1a—the eyespot is not freely accessible from the pipette interior because it sticks to the pipette wall. Then the photoreceptor current has two sources, one being the amplifier, the other being the ground electrode in the bath, together building up a current divider. The relative contribution of these two sources to the photoreceptor current depends on the pipette resistance plus eyespot access resistance on the one hand, and on the resistance between eyespot and ground electrode in the bath on the other hand. As estimated from the position of the eyespot in the pipette, only 50% of the photoreceptor current is supplied from the amplifier. When the eyespot is outside the pipette (b), a capacitive displacement current ($\ominus \rightarrow$), induced by the Ca^{2+} influx, is detectable as an upward signal. The capacitive current amplitude does not depend on the region of the plasma membrane where the current is detected, but on the size of the surface area sucked into the pipette. From micrographs it was estimated to be in the range of 20 to 30% of the total membrane area. Under 'eyespot-in' configuration the capacitance in the pipette (C_1 in c) further reduces the amplitude of the registered photoreceptor current signal, together with the current divider, to a value of about 30–40% of the total. The interpretation of the upward signals as capacitive displacement currents rests on the assumption of a low conductance of the plasma membrane. This appears reasonable for a freshwater alga living in an environment of low ionic strength. c, Simplified electric equivalent circuit of photoreceptor and flagellar currents. G_P , G_L , G_F are the photoreceptor, the leak, and the flagellar conductivities, respectively; C_{1-3} represent parts of the plasma membrane capacitance, and E is the electromotive force for Ca^{2+} , K^+ , and possibly Cl^- . Leakage currents of other ions except K^+ may also contribute to the membrane potential. The numbers 1, 2 and 3 represent the principal measuring positions with their respective pipette seal conductivities (G_S).

response^{1,2}. Flagellar currents occur whenever the photoreceptor current integral oversteps a threshold value. The delay between the two current components (11 to 30 ms) depends on the exposure, this being the shorter the brighter the flash (Fig. 3a). A very similar variation of the delay between flash and response has been observed for the physiological stop response¹⁰. This



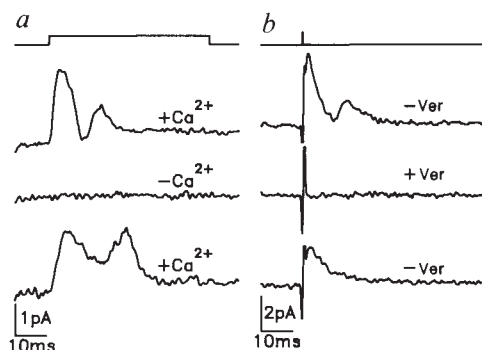


FIG. 4 *a*, Ca^{2+} dependence of the electrical response. Upper trace: control in Ca^{2+} -containing medium ($100 \mu\text{M}$ Ca^{2+} , 5 mM HEPES, 10 mM Cl^- , 1 mM K^+ , adjusted to pH 6.8 with *N*-methyl-D-glucamine); middle trace: the same cell after 5 min perfusion in Ca^{2+} -free solution ($200 \mu\text{M}$ BAPTA instead of Ca^{2+}); lower trace: same cell 5 min after return to Ca^{2+} -containing control medium. Actinic light as in Fig. 1. *b*, Effect of the Ca^{2+} -channel blocker verapamil (Ver). Upper trace: control without verapamil (5 mM HEPES, 10 mM Cl^- , 1 mM K^+ , $300 \mu\text{M}$ Ca^{2+} , $200 \mu\text{M}$ BAPTA, 0.3% dimethylsulphoxide, adjusted with *N*-methyl-D-glucamine to pH 6.8); middle trace: the same cell after 10 min perfusion with control medium containing $600 \mu\text{M}$ verapamil; lower trace: the same cell 10 min after return to control without verapamil. Actinic light as in Fig. 3 (2.4×10^{19} photons m^{-2}).

suggests that the flagellar current is the trigger for the light-induced stop response in a free swimming cell.

Both photoreceptor and flagellar current are suppressed by lowering the Ca^{2+} concentration in the medium and restored when Ca^{2+} is replenished (Fig. 4*a*). They are blocked by verapamil (at $600 \mu\text{M}$; Fig. 4*b*) or pimozone ($20 \mu\text{M}$), two selective inhibitors for L-type Ca^{2+} channels in higher animals¹¹. Both reagents inhibit movement responses of *Chlamydomonas* cells¹², and verapamil inhibits the uptake of $^{45}\text{Ca}^{2+}$ in light¹³. It is therefore reasonable to assume that at least the photoreceptor current is carried by Ca^{2+} . In the case of the flagellar current, a Cl^- efflux cannot be excluded, but Ca^{2+} is considered as a more likely candidate.

Compared with the other signal transduction pathways known in plants, which take from seconds to minutes, ion-channel activation in *Chlamydomonas* is an extremely fast process^{14,15}. It is even faster than the visual process of higher animals. Rapid electrical light responses in a plant system were first recorded from the giant unicellular alga *Acetabularia*^{16,17}, and later from the *Chlamydomonas* relative *Haematococcus*¹⁸. Schilde¹⁶ was probably the earliest to note a similarity between the rapid light responses in a lower plant cell and the photoreceptor potential of vertebrate vision.

On the basis of our results, we propose a basic scheme for behavioural light responses of *Chlamydomonas*: light stimulus $\rightarrow$ rhodopsin activation $\rightarrow$ activation of photoreceptor channels $\rightarrow$ activation of flagellar channels $\rightarrow$ Ca^{2+} increase in the flagellar region $\rightarrow$ flagellar reversal. Intracellular Ca^{2+} can regulate the flagellar beating, as have been demonstrated in permeabilized cells¹⁹. Similar interpretations for signal transduction processes originate in metazoan physiology and have been developed for *Paramecium*, which responds to chemicals, heat, touch and, in some cases, to light²⁰. The evolution of general mechanisms for membrane excitation and sensory transduction apparently pre-dates the separation of plants from animals. □

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A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex

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LIPASES are hydrolytic enzymes which break down triacylglycerides into free fatty acids and glycerols. They have been classified as serine hydrolases owing to their inhibition by diethyl *p*-nitrophenyl phosphate¹. Lipase activity is greatly increased at the lipid–water interface^{2,3}, a phenomenon known as interfacial activation. X-ray analysis has revealed the atomic structures of two triacylglycerol lipases, unrelated in sequence: the human pancreatic lipase (hPL)⁴, and an enzyme isolated from the fungus *Rhizomucor* (formerly *Mucor*) *miehei*⁵ (RmL). In both enzymes the active centres contain structurally analogous Asp-His-Ser triads (characteristic of serine proteinases), which are buried completely beneath a short helical segment, or 'lid'. Here we present the crystal structure (at 3 Å resolution) of a complex of *R. miehei* lipase with *n*-hexylphosphonate ethyl ester in which the enzyme's active site is exposed by the movement of the helical lid. This movement also increases the nonpolarity of the surface surrounding the catalytic site. We propose that the structure of the enzyme in this complex is equivalent to the activated state generated by the oil–water interface.

Many factors have been suggested to trigger the activation of lipase at a lipid–water interface. They included increase of substrate concentration at the interface⁶, better orientation of the scissile ester bond⁷, reduction in the water shell around the ester molecules in water⁸ and a conformational change in the enzyme^{2,9}. To investigate both the nature of the catalytic interactions and the structural changes associated with lipase activation, the enzyme was reacted with a series of substrate analogues. We hoped that the enzyme would be trapped in the activated state allowing the structural changes to be identified. Often long incubation times or high concentrations of organic solvents were needed for reaction. One compound, *N*-hexyl chlorophosphonate ethyl ester, reacted stoichiometrically and inhibited the enzyme fully and irreversibly. Crystals of this complex grew as

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FIG. 1 *a*, Stereo figure showing the difference electron density for the inhibitor in the active site of RmL. The map is calculated with coefficients $|F_{\text{obs}}| - |F_{\text{calc}}|$ and is phased on protein atoms only. Two levels of density are shown: 3 and 10 r.m.s. deviations from the mean. The phosphorus atom is associated with the highest positive density of the difference map. The model of *n*-hexylphosphonate ethyl ester was built into the density, but not refined. Only selected residues lining the binding and catalytic sites are shown. The fatty acid binding site is considerably longer than the six-carbon aliphatic chain of the inhibitor. The catalytic serine is immediately beneath the phosphorus atom of the inhibitor, and is not shown in this view. His 258 is clearly displaced towards the ethyl oxygen, consistent with the proposed orientation of the substrate and the mechanism of hydrolysis. Hydrogen-bond contacts between Ser 82 O_γ and its amide NH are indicated. *b*, Cross-section through the inhibitor and its electron density, illustrating the interactions with Ser 82, Ser 144 and His 257. The electron density is consistent with the proposed tetrahedral stereochemistry of the complex. The contour levels are the same as in *a*. The Ser O8—P bond is not drawn. *c*, Stereo figure showing the electron density for the final model of the central part of the lid in its open conformation. This map has been calculated with coefficients $2|F_{\text{obs}}| - |F_{\text{calc}}|$ and phased on the complete protein model. A single arbitrarily selected contour level is shown. There is no density which could be associated with the lid in its closed, or native, conformation.

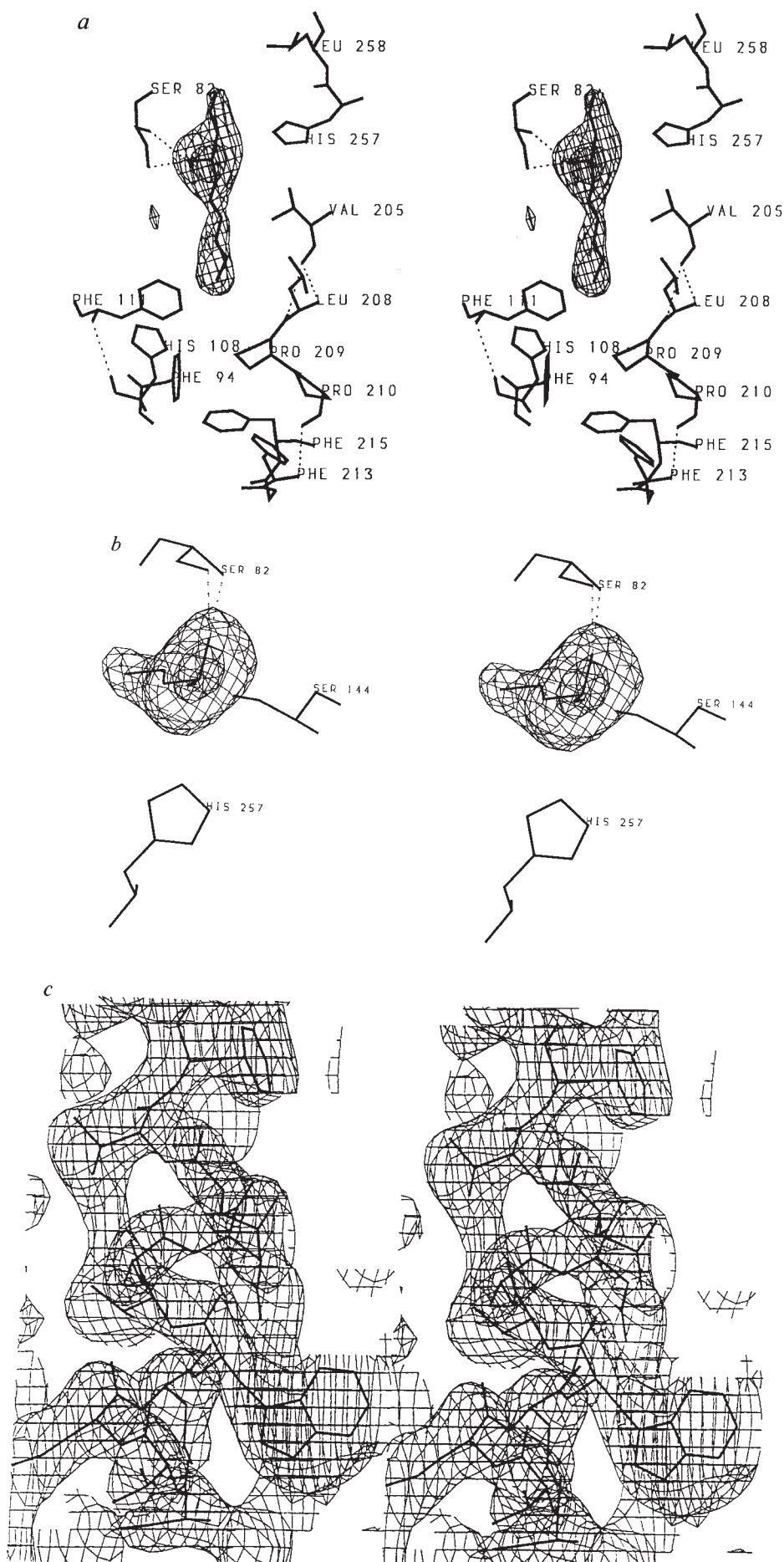


TABLE 1 Data set obtained by crystallography

Resolution limit (Å)	No. of reflections possible	No. of reflections collected (%)	Average redundancy	No. of singly measured reflections (%)	$\langle 1/\sigma(I) \rangle$	No. of reflections > 2 σ (%)	R_m (%)
<5.44	1,043	99.0	7.2	1.1	89.9	96.2	5.1
<4.32	982	100.0	7.7	0.3	68.4	96.5	6.5
<3.77	967	100.0	8.1	0.2	48.1	94.0	8.5
<3.43	974	99.7	6.9	0.6	32.5	92.9	10.3
<3.18	956	96.4	4.3	9.7	14.5	85.1	16.1
<3.00	965	27.1	2.0	43.8	6.5	61.8	18.6
Total	5,892	87.3	6.6	4.4	49.3	91.5	7.9

R_{m} , Conventional crystallographic merging R factor (on intensities, I). σI is the associated standard deviation. The crystals of the complex were prepared as follows. Purified recombinant RmL^{16.17.18} was dissolved in 20 mM Tris-HCl buffer (pH 8.05) to a concentration of 1 mg ml⁻¹; *n*-hexylchlorophosphonate ethyl ester was prepared according to Fukuto and Metcalf¹⁹. It was dissolved in dimethylsulphoxide (DMSO); 10–100 mM stock solutions were used. They were added to the enzyme solutions and incubated overnight at 37 °C. The final DMSO concentration did not exceed 5% (v/v). Any precipitated protein was removed by centrifuging at 15,000*g*. The supernatant was concentrated, and washed three times with the Tris-HCl buffer. The final protein concentration was ~20 mg ml⁻¹. Crystals were grown by the hanging drop method; 7–10% poly(ethylene glycol)(*M*, 600) was used as a precipitant. The crystals grew in the form of clusters of very thin (0.01–0.05 mm) and fragile plates. They have the symmetry of space group C222₁, with axial lengths $a = 48.3$ Å, $b = 93.9$ Å, $c = 122.1$ Å. The native RmL crystals are in the space group P2₁2₁2₁ with axial lengths $a = 71.6$ Å, $b = 75.0$ Å and $c = 55.0$ Å⁵. **Data collection and processing:** X-ray data were collected using the Xentronics (Siemens) area detector mounted on a conventional source ($\lambda = 1.5418$ Å, fine focus tube operated at 50 kV and 25 mA, graphite monochromator) and positioned 10 cm from the sample²⁰. Data were processed using the XGEN suite of programs²¹. **Structure solution and crystallographic refinement:** The structure was solved by the molecular replacement method, using the refined coordinates of the native enzyme and the CCP4 crystallographic software package²². The rigid body parameters were refined using the protocol of Derewenda²³, which reduced the initial crystallographic R factor ($\sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}}$) for the unrefined model (all atoms except the lid) from 0.52 to 0.36. The Konnerth-Hendrickson restrained refinement method²⁴ and the X-plor method which includes simulated annealing²⁵ were used to refine the model. The missing residues of the lid were built manually using the FRODO program²⁶ and the Evans and Sutherland PS330 graphics display system. There was no density in the position occupied by the lid residues in the native enzyme. Final refinement included all protein atoms, but excluded the inhibitor and solvent; the final R factor was 0.185. **Structural comparisons:** The nature of the conformational change was analysed after the enzyme and the enzyme-complex coordinate sets were superimposed by the method of least-squares, using the main-chain atoms of all amino acids except those in the lid.

thin and extremely fragile plates and in a different space group from that of the native enzyme. These crystals mostly diffract very poorly but it was possible to collect a 3 Å data set from a single crystal (Table 1). It was not possible to extend the resolution with synchrotron radiation as the crystals available failed to diffract satisfactorily. The initial electron density map showed that the helical lid (residues 85–92) had moved, exposing the active site containing the inhibitor. After refinement (see legend to Fig. 1) the electron density for the inhibitor and for the newly positioned lid was well defined and easily interpreted.

An atomic model of the inhibitor could be built unambiguously into the active site (Fig. 1*a*), since the electron density corresponding to both the ethyl and *n*-hexyl moieties was clearly defined. A direct covalent bond was formed between the nucleophilic O γ of Ser 144 and the phosphorus atom of the inhibitor. The ester carbonyl oxygen seems to be stabilized by the interactions with the peptide amide nitrogen and the hydroxyl of Ser 82 (Figs. 1*a, b* and 2). The main-chain amide nitrogen of Leu 145 may also be involved but higher resolution data is needed to resolve this. Comparison with the native structure⁵ shows that the Ser 82 side chain assumes a favourable conformation for oxy-anion interaction only after the lid has moved away from the active site. The formation of oxy-anion contacts by Ser 82 in the complex suggests that the full, or nearly

full, extent of the lid's rigid body movement in activation has taken place. The *n*-hexyl moiety lies in a hydrophobic groove created partly by the displacement of the lid but also partly preexisting in the native enzyme. This groove may normally be occupied by the leaving fatty acid cleaved from the triglyceride. The ethyl group lies in the position of the leaving diacylglyceride, but as it lacks the two relevant ester groups present in the triacylglycerol substrates, no conclusions can be drawn about their interactions in the binding pocket.

Because the chemistry of ester and amide hydrolysis is very similar¹⁰ the structure of the inferred tetrahedral intermediate complex is reminiscent of that found in serine proteinases (Fig. 2). There is, however, the structural difference that in serine proteases the interactions at the oxy-anion hole are preformed whereas in this lipase they are generated by the activation process.

The conformational change of the lid can be described as a simple rigid body movement of its helical part (residues Leu 85-Asp 91). It consists of a translation of the centre of gravity of about 8 Å and a rotation of 167° about an axis almost parallel to that of the helix (Fig. 3*a, b*). There are two clearly defined hinge regions, the serine tripeptide 82-84 and the tetrapeptide 92-95, one on each side of the lid, which allow this movement. As the helical lid rolls back from the active site, its hydrophilic side, which is exposed to the solvent in the native structure, becomes partly buried in a polar cavity previously filled by well-ordered water molecules. At the same time the hydrophobic side of the lid becomes completely exposed, greatly expanding the nonpolar surface around the active site (Fig. 3*c, d*). Thus the exposure of the catalytic residues is accompanied by a marked increase in the nonpolarity of the surrounding surface. Interfacial activation is thus explained by the stabilization of this nonpolar surface by the lipid environment which would in effect create a catalytically competent enzyme, able to attack the triglyceride molecules within the lipid phase.

The enzyme phospholipase A2 also exhibits interfacial activation, although the mechanism is quite different¹¹ and there appear to be no main chain movements when the substrate is bound¹²⁻¹⁴. In contrast to the fungal lipase the catalytic surfaces of phospholipase A2 are exposed in aqueous conditions and on

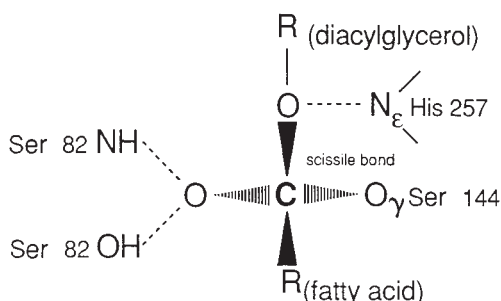
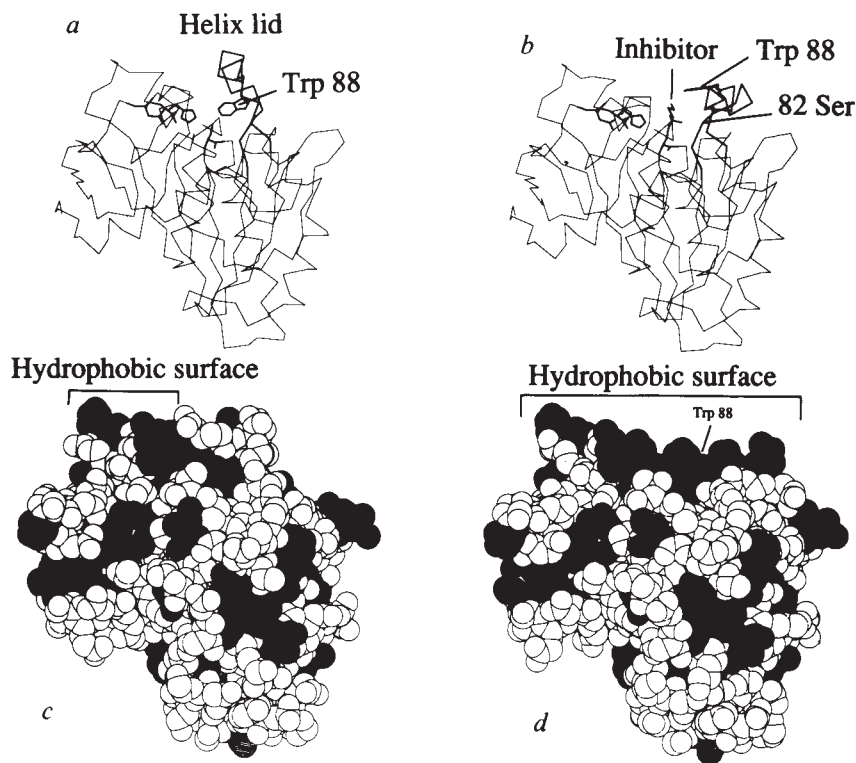


FIG. 2 Schematic representation of the inferred tetrahedral enzyme-substrate complex of RmL.

FIG. 3 The interfacial activation of lipase. Diagrammatic representation of the conformational change in RmL. The view is in a direction near to the axis of the helical lid and is approximately perpendicular to the view in Fig. 1a. The native enzyme (a) and the complex (b) are drawn with their C_{α} backbone. The catalytic triad, the tryptophan 88 and the lid, and the inhibitor are drawn with all atoms and highlighted with thick bonds. The complete atomic structures drawn with their van der Waals' radii of the native molecule (c) and the complex (d). The nonpolar atoms are shaded. The increase in the extent of the nonpolar surface is readily seen in this view. The inhibitor is masked by the tryptophan 88 of the lid and sidechains lying on the nearside of the molecule.



substrate binding there seem to be no significant shifts in the positions of the main-chain atoms, only side-chain movements and the repositioning of a calcium ion. Activation takes place when the lipid bilayer forms around the active site. The stacking of the phospholipid against the enzyme presents the individual molecules to the active site in the correct conformation for binding and cleavage. The bilayer also excludes water and thus removes the need to solvate the lipid during transport between its micelle and the enzyme. Thus phospholipase activation in this enzyme seems to be generated directly by the shielding and conformational properties of the lipid bilayer. In *R. miehei* lipase, however, it is achieved by the displacement of a lid structure whose movement exposes the catalytic groups and creates a nonpolar surface which stabilizes the contact between the enzyme and the lipid interface. A similar mechanism may work in the human pancreatic lipase⁴ where there is also a helical lid containing tryptophan sitting over the active site. Here, however, other structural changes are thought to be needed for access to the catalytic pocket⁴. The burial of the active site in a pancreatic and a fungal lipase suggests this may be a general feature in this class of enzyme. It therefore follows that interfacial activation in lipases will generally be associated with specific structural changes at the active site, in contrast to phospholipase A₂. □

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Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor

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THE transforming proteins of several DNA tumour viruses, including adenovirus E1a and simian virus 40 large T antigen, complex with the retinoblastoma (Rb) tumour-suppressor gene product^{1,2}. This requires regions in these viral proteins necessary for transformation and is thought to inactivate the growth-suppressing properties of the Rb protein by disrupting its interaction with cellular targets³. Indeed, regions of Rb required to form a complex with E1a and large T antigen are often mutated in transformed cells⁴. The level at which the Rb protein regulates proliferation is unknown, although one possibility is transcription. We have previously characterized a sequence-specific transcription factor, DRTF1, the activity of which is downregulated as embryonal carcinoma stem cells differentiate. DRTF1 is found in several discrete protein complexes (a, b and c) which are of different sizes but have the same DNA specificity^{5,6}. We now show that one of

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these also contains the Rb protein and, further, that the adenovirus E1a protein causes the dissociation of the Rb protein from this complex. This requires conserved regions 1 and 2 of E1a that are known to be required for efficient transformation⁷. These results demonstrate that the Rb protein forms a complex with a DNA-bound transcription factor, and suggests that the Rb protein might act by regulating transcription.

In F9 EC cell extracts the 1b/c form of DRTF1 (from now on referred to as 1b) is more abundant than the 1a form⁸. But as F9 embryonal carcinoma (EC) stem cells differentiate to F9 parietal endoderm-like cells (F9PE), DRTF1b is strongly down-regulated, whereas DRTF1a becomes the predominant form (J.F.P. and N.B.L.T., manuscript in preparation). DRTF1a can be dissociated into 1b by a variety of treatments (J.F.P. and N.B.L.T., manuscript in preparation), suggesting that DRTF1a contains the 1b DNA-binding activity and another polypeptide(s). DRTF1 binds to the same motif as the HeLa cell transcription factor E2F, although the relationship between DRTF1 and E2F is unclear.

Two cell lines derived from human leukaemias, JM and H9, were found to contain DRTF1a in abundance, but little 1b (Fig. 1a, compare lanes 1, 2 and 3). These activities had the expected DNA-binding specificity⁸ but slightly different mobilities to the F9 1a form (Fig. 1a and b); JM cell extracts were used to characterize further the DRTF1a activity.

The downregulation of DRTF1 during F9 EC cell differentiation correlates with the degree of cellular E1a-like activity⁵, originally defined by the ability of F9 EC cells, but not their differentiated derivatives, to allow the expression of an adenovirus deletion mutant which lacks a functional viral E1a gene⁹. Because DRTF1b is abundant in F9 EC cells where there is an endogenous cellular E1a-like activity, we tested whether 1a was modulated by viral E1a. The mobility of DRTF1a in F9

EC, F9 PE and JM cell extracts increased to that of 1b after the addition of adenovirus E1a (Fig. 2a). This effect was specific for DRTF1 as E1a did not affect the binding of ECRE2 (ref. 13) to the ATF DNA-binding site (Fig. 2a). This could mean that the adenovirus E1a protein sequesters a protein(s) from the 1a complex to produce the 1b complex. That 1b is abundant and 1a scarce in F9 EC cell extracts is consistent with the presence of a cellular E1a-like activity that acts analogously to viral E1a.

The adenovirus E1a gene is differentially spliced to produce mRNAs whose protein products have distinct biological properties⁷. Both the products of the 12S and 13S coding sequence contain two regions, known as conserved regions (CR) 1 and 2 (Fig. 2c), which are required for E1a to bind several cellular polypeptides, including the Rb protein^{4,10}, and for immortalization and cooperation with other oncogenes⁷. Both 13S- (Fig. 2a and b) and 12S- (Fig. 2b) encoded polypeptides dissociate 1a, but two 12S mutants with deletions in either CR1 (Sp53) or CR2 (SpCS) (Fig. 2c) failed to cause the dissociation of 1a (Fig. 2b), suggesting that both CR1 and CR2 are required for this. As these conserved domains in E1a are also required for binding cellular proteins, we reasoned that E1a may act to sequester a cellular protein from the 1a complex.

A candidate for this cellular protein was the Rb protein, and to test this we assayed a number of different monoclonal antibodies that react with distinct epitopes in the Rb protein (ref. 1; Q. Hu and E. Harlow, manuscript in preparation) for their ability to super-shift the DRTF1a-complex, which would indicate the presence of Rb in the DNA-bound protein complex. Two monoclonal antibodies, C36 and XZ55, which react with different regions of the Rb protein, each produced a super 1a-shift (Fig. 3a, lanes 4 to 7, and 10 to 13), confirming that the Rb protein is indeed present in DRTF1a. A control monoclonal

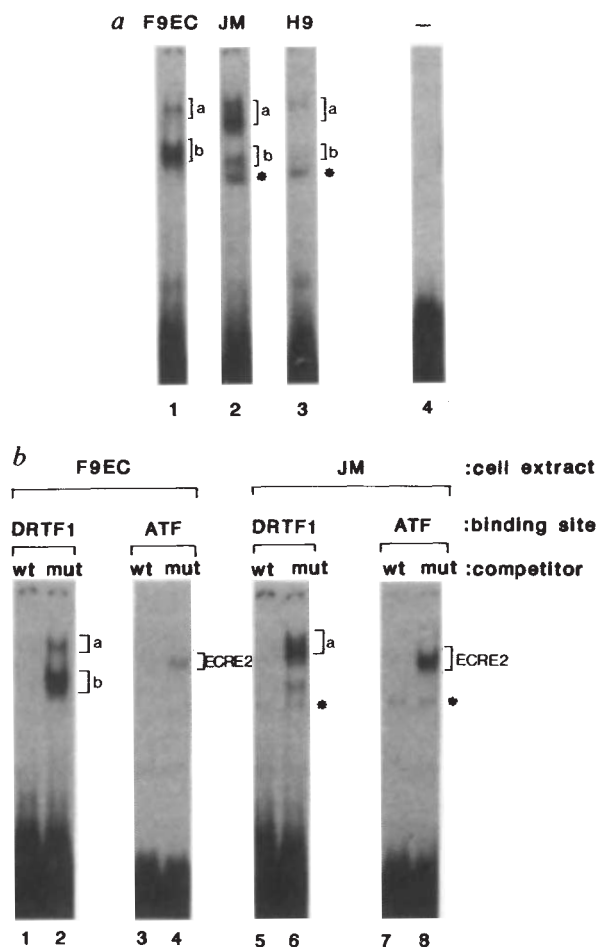


FIG. 1 a, Distinct forms of DRTF1 in different cell-types. Complexes formed on a probe containing a DRTF1 site in F9 EC (lane 1), JM (lane 2) and H9 (lane 3) cell extracts; complexes a and b are indicated. Lane 4 shows the probe alone, and the asterisk indicates a non-specific complex. b, Sequence specificity of DRTF1 complexes in F9 EC and JM cell extracts. Specificity of the complexes formed on the probe containing the DRTF1-site was determined by competition with oligonucleotides containing either wild-type (lanes 1 and 5) or mutant (oligonucleotide 62/60, lanes 2 and 6) DRTF1 binding sites; DRTF1 complexes are indicated. As a control, a similar assessment of complex specificity was performed with a probe containing an ATF site⁸, using either a wild-type (wt; lanes 3 and 7) or mutant (mut; lanes 4 and 8) ATF-site as competitor; the ATF-site binding activity, ECRE2, is indicated. METHODS. Gel retardation assays were performed as described previously⁸ in F9 EC, JM or H9 cell extracts prepared according to standard procedures. The wild-type DRTF1 binding site probe contains sequences from the adenovirus E2A promoter -71 to -50 (ref. 8), and the mutant 62/60 has sequences -62 to -60 mutated⁸. Probe P contains a wild-type ATF binding site taken from the adenovirus E4 promoter¹³; Pm1, is a mutant ATF site, unable to bind ATF (ref. 13).

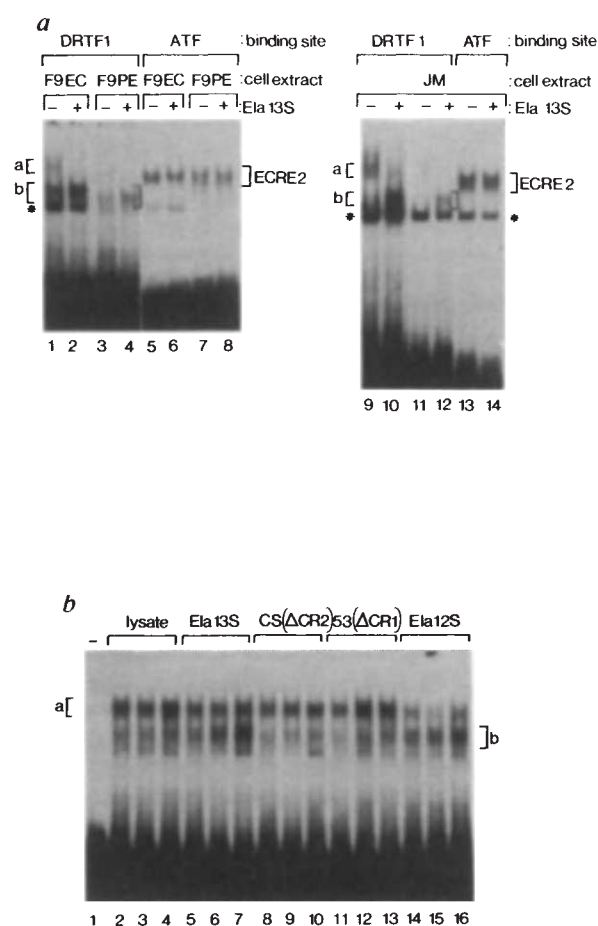


FIG. 2 Adenovirus E1a dissociates DRTF1a. **a**, The 13S E1a coding sequence was transcribed, translated and assessed for its effect on DRTF1 in either F9 EC (lanes 1 and 2), F9 PE (lanes 3 and 4) or JM cell extracts (lanes 9 to 12). The 13S E1a product dissociated the 1a complex in all three cell extracts (lanes 2, 4, 10 and 12) to produce the faster migrating 1b-complex whereas the control lysate containing translated BMV RNA did not (lanes 1, 3, 9 and 11); lanes 11 and 12 show a short exposure of lanes 9 and 10. The E1a 13S product had no effect on the binding activity of ECRE2 to the ATF site in any of the extracts studied (lanes 5 to 8, 13 and 14). **b**, The indicated coding regions of E1a were transcribed, translated and assessed for their ability to dissociate the 1a complex in the JM extract. Both 13S (lanes 5 to 7) and 12S E1a (lanes 14 to 16) dissociated 1a (compare the induction of 1b to the lysate alone; lanes 2 to 4), whereas Sp52 (ΔCR1) and SpCS (ΔCR2) did not (lanes 8 to 13). Each series of lanes shows the results of titrating in equal amounts of lysate, with increasing concentration from left to right. The reticulocyte lysate alone was titrated in lanes 2 to 4. **c**, Summary of coding sequences and deletions. The shaded regions in E1a and derivatives indicate CR1 and CR2.

METHODS. All coding sequences were cloned into Sp65 and transcribed and translated by standard procedures. Translation efficiency was assessed by examining the polypeptides on standard SDS-polyacrylamide gel electrophoresis before they were used in gel retardation experiments. Translated polypeptides were added to the JM cell extract before addition of the binding site; binding site probes are as used in Fig. 1.

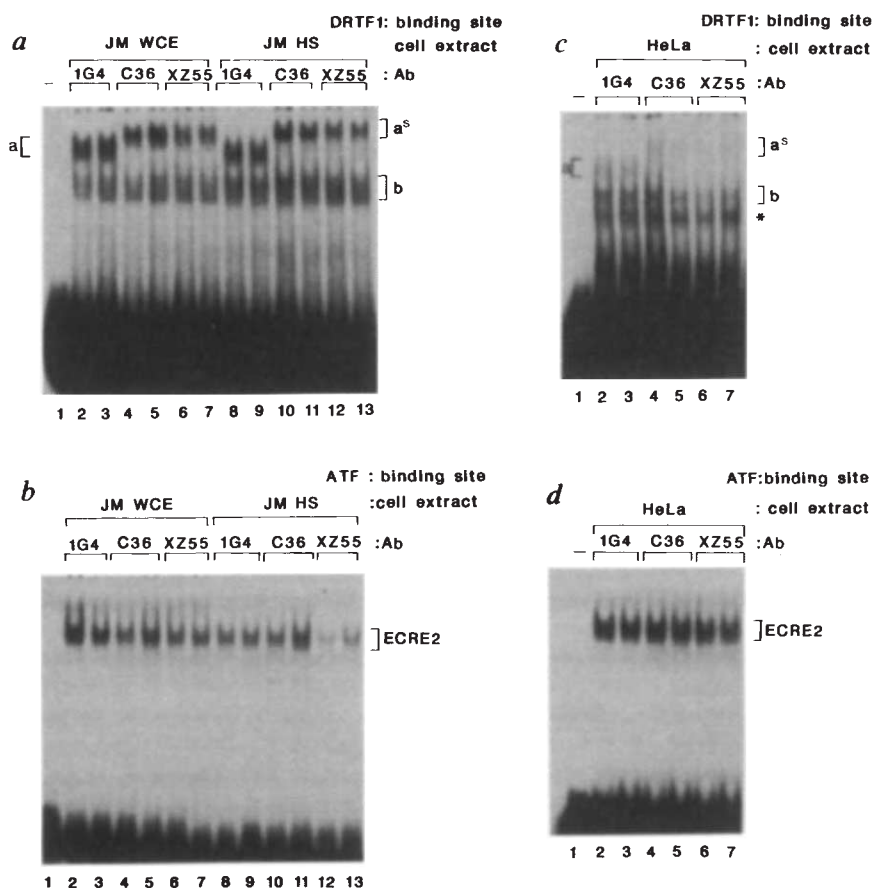


FIG. 3 The retinoblastoma gene product complexes with DRTF1. **a**, Monoclonal antibodies against the Rb protein were added to either JM (**a** and **b**) or HeLa (**c** and **d**) whole cell extracts (WCE) and assessed by gel retardation using either a DRTF1 (**a** and **c**) or an ATF (**b** and **d**) binding site. Shifted-shifts are indicated in JM extracts (**a**) and the much weaker shifted complex in HeLa cell extracts (**c**) as **a**^s. Note that the 1b complex in HeLa and JM cell extracts is unaffected by the anti-Rb antibodies, as is the ECRE2 complex formed in both cell-types on the probe containing the ATF site. A heparin-Sepharose fraction from JM cell extracts (HS) was used in lanes 8 to 13.

METHODS. Monoclonal antibodies C36 and XZ55, which react with different regions of the Rb protein (1; Q. Hu and E. Harlow, manuscript in preparation), were added to the extract and incubated for 10 min before the addition of the appropriate binding site. As a control, a monoclonal antibody, 1G4, which reacts with an unrelated antigen¹² but has the same isotype as C36 and XZ55, was used. Binding site probes are as in Fig. 1.

antibody that reacts with an unrelated protein¹² had no effect on the 1a-shift (Fig. 3a, lanes 2 and 3, and 8 and 9). Also, the super-shift was specific for DRTF1 because the ATF activity ECRE2 was unaffected by the antibodies (Fig. 3b). This clearly establishes that the Rb protein is present in DRTF1a, and strongly argues that E1a acts to sequester Rb from the complex. If this is the case, then DRTF1b should not be affected by the anti-Rb antibodies because this form is generated by E1a (Fig. 2a). This prediction was tested in a HeLa cell extract which contains predominantly the DRTF1b form⁸ (possibly because HeLa cells express the human papilloma virus HPV-18 E7 protein). As predicted, this complex was unaffected by the anti-Rb antibodies, although low amounts of a shifted-shift were visible with C36 and XZ55 (Fig. 3c, lanes 4 to 7), most probably derived from the small amount of 1a in HeLa cell extracts⁸. Similarly, the small amounts of 1b in the JM cell extract was not affected (Fig. 3a). We conclude from these studies that the Rb gene product when complexed with DRTF1 produces 1a and that adenovirus E1a acts to disrupt this complex by sequestering the Rb protein.

Although the mechanism by which the Rb protein negatively regulates cellular proliferation is unknown, it is clear that it binds a number of cellular proteins which might be involved, an idea supported by the inability of some naturally occurring Rb mutants to bind these activities³. Here we have demonstrated that the Rb protein is complexed to a cellular transcription factor, thereby enabling the Rb protein to localize to DNA. As DRTF1 is a transcriptional activator, it is possible that by complexing with it, the Rb protein regulates this activity. Indeed, the transcriptional activity of affinity-purified DRTF1a is severely compromised when assayed by *in vitro* transcription (data not shown).

This provides a molecular explanation of how E1a, and other

viral transforming proteins such as simian virus 40 large T antigen, might in part act: by forming a complex with Rb they inactivate the transcription-repressing properties of the Rb protein and thereby indirectly activate transcription of those genes normally subject to Rb-mediated negative regulation. Similarly, mutations that occur in the Rb gene in a variety of transformed cells are often found to modify the region known to bind E1a⁴ and might interfere with the transcription properties of the Rb protein. It will be interesting to establish whether these mutant Rb genes encode proteins that can complex with DRTF1.

Finally, certain stem cells, F9 EC for example, have a cellular E1a-like activity which reduces the level of the DRTF1/Rb complex. Presumably a cellular E1a-like activity functions to regulate the association of the Rb protein with cellular transcription factors such as DRTF1. This activity might be important in regulating the proliferation of stem cells. □

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A search for the most stable folds of protein chains

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It is generally believed that it is not sensible to search for a thermodynamically stable structure of a protein^{1,2} because neither a molecule nor a computer can look through all the 3^{100} possible (for 100 residues) chain conformations. Here we show that the use of a molecular field theory for the long-range interactions, the use of one-dimensional statistical mechanics for the short-range ones and the discovery that there are^{3,4} and there must be^{5,6} only a small discrete set of folding patterns, make it possible to examine all the variety of 'potentially stable' structures. The general approach and its application is demonstrated here by calculation of stable folds for some β domains. The most stable of these folds correspond to the observed structures.

Contrary to common opinion, the multitude of structures by itself is no obstacle to finding a stable fold. If particles interact only with neighbours in a chain, as in Zimm-Bragg's model⁷, a general recursive algorithm^{8–10} of one-dimensional statistical mechanics can find the average position in space for each of them. This is true even if the particles also interact with an external force-field¹⁰, regardless of its complexity.

The real difficulty is caused by the many different interactions between remote chain regions. Problems of multiparticle interaction are common in statistical physics, and are usually overcome using a molecular field approximation¹¹. This helps to find the overall properties of magnets¹¹, polymers¹⁰, spin glasses¹² and

to examine denaturation of proteins^{13,14}. The molecular field method has been applied recently^{15–18} to the analytical investigation of the problem of a 'unique' chain-fold selection. The approximation replaces the actual energy $\sum_{j \neq i} \epsilon_{i,j}(r_i, r_j)$ of the interactions between a particle i and other ones by a mean 'molecular field potential' $\phi_i(r_i)$ acting at this particle at a space point r_i .

A similar approach is used here to find a stable protein fold. For a polymer, only the long-range interactions, $\epsilon_{i,j}$, need to be approximated by a molecular field, but we must consider explicitly the short-range ones, $u_{i,i+1}$, which include covalent binding¹⁰. As a result, the actual energy

$$E(r_1, \dots, r_N) = \sum_i \xi_i(r_i) + \sum_i u_{i,i+1}(r_i, r_{i+1}) + \frac{1}{2} \sum_{i \neq j} \epsilon_{i,j}(r_i, r_j) \quad (1)$$

(where ξ_i is the internal energy of particle i) is approximated as

$$E \approx \tilde{E}(r_1, \dots, r_N) = \sum_i \xi_i(r_i) + \sum_i u_{i,i+1}(r_i, r_{i+1}) + \sum_i \phi_i(r_i) - C \quad (2)$$

The molecular field potentials ϕ are estimated¹¹ as

$$\phi_i(r_i) = \sum_{j \neq i} \left[\sum_{r_j} \epsilon_{i,j}(r_i, r_j) W_j(r_j) \right] \quad (3)$$

$W_j(r_j)$ is a probability for a particle j to be in a position r_j , and $C = \frac{1}{2} \sum_i \sum_j \phi_i(r_i) W_j(r_j)$ does not depend on coordinates and accounts for the fact that each $\epsilon_{i,j}$ contributes to both ϕ_i and ϕ_j . One can see that equations (2) and (3) are precise for fixed structures (where $W_j(r_j) \neq 0$ only in one point) and applicable

to fluctuating ones, independently of what forces contribute to ϵ . To describe a fold, we have to find its partition function Z and an average position of each particle, $\langle r_i \rangle = \sum_r r_i W_i(r_i)$.

With long-range interactions replaced by molecular field potentials, the calculation of a three-dimensional chain fold is mathematically equivalent to the search for helices in heteropolypeptides. One need not add all terms one by one to calculate exactly: (1) the partition function of all chain conformations

$$Z = \sum_{r_1} \cdots \sum_{r_N} \exp[-\tilde{E}(r_1, \dots, r_N)/RT] \quad (4)$$

(T , temperature; R , gas constant; sum is taken over all positions of all particles); and (2) the spatial distributions

$$\tilde{W}_i(r_i) = \frac{1}{Z} \sum_{r_1} \cdots \sum_{r_{i-1}} \sum_{r_{i+1}} \cdots \sum_{r_N} \exp[-\tilde{E}(r_1, \dots, r_N)/RT] \quad (5)$$

The sum is a partition function of the chain with a particle i fixed in position r_i . This sum is a product (compare with equations (2), (4) and (5)) of two terms: $p_i(r_i)$, a partition function of the chain fragment preceding this particle i , and $q_i(r_i)$, a partition function of the following chain fragment. One can compute p_i and q_i using the fast recursive algorithm⁹, although r_i stands now for composite multidimensional coordinates (see below) rather than for dihedral angles, and the 'particles' in this case are β strands rather than residues:

$$\begin{aligned} p_1(r_1) &\equiv 1 \\ p_{i+1}(r_{i+1}) &= \sum_{r_i} p_i(r_i) \exp[-(u_{i,i+1}(r_i, r_{i+1}) \\ &\quad + \phi_i(r_i) + \xi_i(r_i))/RT] \quad 1 \leq i < N \\ q_N(r_N) &= \exp[-(\phi_N(r_N) + \xi_N(r_N) - C)/RT] \\ q_i(r_i) &= \sum_{r_{i+1}} q_{i+1}(r_{i+1}) \exp[-(u_{i,i+1}(r_i, r_{i+1}) \\ &\quad + \phi_i(r_i) + \xi_i(r_i))/RT] \quad N > i \geq 1 \end{aligned} \quad (6)$$

Sums are taken over all positions of a particle i or $i+1$. These relations give vectors p_{i+1} as a function of p_i (as i increases from 1 to N , more residues contribute to the vector) and q_i as a function of q_{i+1} (more residues to contribute to this vector as i decreases from N to 1). Then

$$Z = \sum_{r_1} p_1(r_1) q_1(r_1) = \cdots = \sum_{r_N} p_N(r_N) q_N(r_N) \quad (7)$$

and

$$\tilde{W}_i(r_i) = p_i(r_i) \cdot q_i(r_i) / Z \quad (8)$$

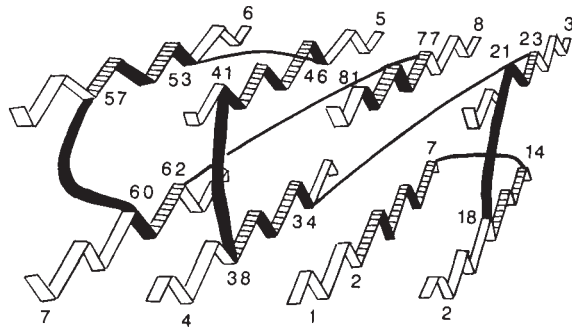


FIG. 1 Three-dimensional lattice model for the β -sandwich fold of γ -crystallin first domain²², shown as a path of protein chain through this lattice. The native location of β strands in space and in the sequence is indicated; the loops are presented schematically. The lattice allows for excluded volume of the β strands and for hydrogen-bonds between them. For simplicity, the β sheets are not twisted and β bulges are not shown. 'In' and 'out' pleating of the lattice corresponds to 'in' and 'out' positions of side chains. The coarse features of a hydrophobic molecular field arise from the folding pattern: the field is stronger for the residues on the inner β strands (1, 4, 5, 8) and weaker for the edge ones (2, 3, 6, 7).

A thermodynamically stable or metastable structure is 'self-consistent' with a molecular field produced by itself¹¹: the values of $\tilde{W}_i$ obtained from equations (6)–(8) will converge to match the W_i values which had been used to calculate the ϕ values. Until $\tilde{W}_i$ and W_i coincide, we use $W_i^{\text{eff}} = \lambda \tilde{W}_i + (1 - \lambda) W_i$ (where $\lambda \approx 1/2$; manuscript in preparation) in equation (3). This is an iteration procedure; it begins with some 'starting' ϕ_i (for example $\phi_i \equiv 0$) and continues until a self-consistent solution is obtained. There may be a set of metastable solutions depending on the region of conformational space ('folding pattern') and the 'starting' potentials¹¹; a stable one corresponds to the maximum partition function.

We consider the β -sandwich protein folds taking into account only the main long-range interactions: steric repulsion (excluded volume), backbone-backbone hydrogen-bonds and hydrophobic forces. Close packing is not considered, which means that we are dealing with something more like a fluctuating 'molten' globule^{14,19} than a native 'solid' protein.

In searching for the most stable fold of a chain we need to examine only the set of 'potentially stable' folding patterns or topologies. These patterns correspond to a low free energy of sequence-independent interactions⁵. They look like compact packings of α and β segments; the segments are connected by loops which have a definite chirality and cross neither each other nor the sides of the layers (Fig. 1). This set is very limited: there are, for example, only 60 'stable' patterns among $\sim 10^5$ different ones which correspond to a sandwich with four anti-parallel β strands in each sheet⁵.

The program²⁰ considers the all- β stable folding patterns. Here a 'particle' is a β strand and i ranges from 1 to 8. A 'coordinate' r_i includes: (1) n_i , sequence number of first residue in strand i ; (2) x_i, y_i, z_i , lattice coordinates of that residue; (3) l_i , length of this strand i . When the program starts to work, it 'knows' only the pattern (x_i, z_i , and direction for each strand) but it must search n_i, l_i and y_i . Each pattern includes a lot of conformations: there are $\sim 10^2$ possible values for each n_i (this range is determined by chain length), ~ 10 for each l_i (permitted

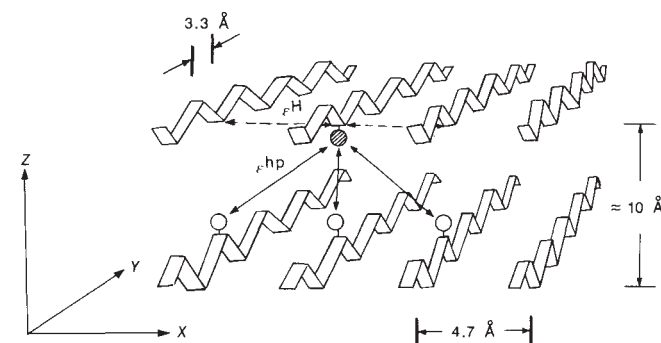
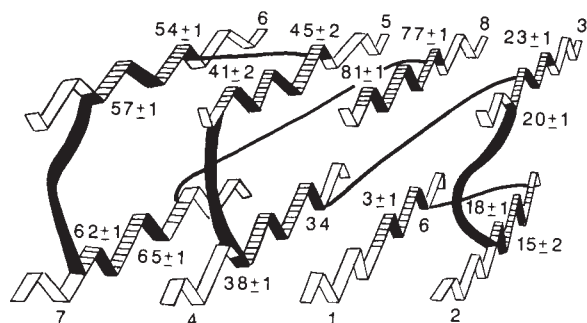


FIG. 2 Interactions within the lattice. For one residue (striped), all permitted long-range contacts within the sheet (dashed arrows) and with the other β sheet (solid arrows) are indicated. The intrinsic energy of any β strand is the sum of the energies of the residues: $\xi = \sum \Delta \xi_i$; $\Delta \xi = F_\beta + 2\Delta F_e$, F_β being a free-energy difference between interior of β -sheet and a coil and ΔF_e an additional energy of a residue at a β -sheet edge²³. Thus, the energy of intrasheet contact is $\epsilon^H = -\Delta F_e' - \Delta F_e''$ (primed quantities refer to residues on strand i and double primed to residues on other strands). For 'in' residues, the energy of intersheet contacts $\epsilon^{hp} = \frac{1}{6}(\Delta G_{hp}' + \Delta G_{hp}'')$, ΔG_{hp} being hydrophobic free energy of transfer value²⁴. For 'out' residues, ϵ^{hp} is taken to be $\frac{1}{6}2\Delta G_{hp}^{\text{Ala}} = -0.13 \text{ kcal mol}^{-1}$. For intrasheet interaction of β strands, $\epsilon_{ij} = \sum \epsilon^{ij}$ (the sum includes all contacts between the residues of the strands i and j), and $\epsilon_{ij} = \sum \epsilon^{hp}$ for intersheet interaction. In result of averaging equation 3, molecular field potential for a β strand has a form $\phi_i = \sum_{\text{in+out}} [-\Delta F_e' - \langle \Delta F_e'' \rangle] \langle n_i^H \rangle + \sum_{\text{in}} \frac{1}{6}[\Delta G_{hp}' + \langle \Delta G_{hp}'' \rangle] \langle n_i^{hp} \rangle + \sum_{\text{out}} \frac{2}{6}\Delta G_{hp}^{\text{Ala}} \langle n_i^{\text{Ala}} \rangle$. The sum is taken over all 'in' and 'out' residues of a strand i . $\langle n_i^H \rangle$ is the averaged amount of intrasheet neighbours of a lattice point occupied by a residue; $\langle \Delta F_e'' \rangle$ is average edge energy of these neighbours. $\langle n_i^{hp} \rangle$ and $\langle \Delta G_{hp}'' \rangle$ are average amounts of the neighbours on the opposite β sheet and their average hydrophobicity. One can see that $0 \leq \langle n_i^H \rangle \leq 2$ and $0 \leq \langle n_i^{hp} \rangle \leq 3$ for inner β strands while for the edge ones $0 \leq \langle n_i^H \rangle \leq 1$ and $0 \leq \langle n_i^{hp} \rangle \leq 2$.



strand length is 1 to 11 residues) and ~ 5 for each y_i . Thus, there are $\sim (10^2 \times 10 \times 5)^8$ what is $\sim 10^{30}$ different structures (in fact $\sim 10^{20}$ – 10^{25} , because the fluctuations are not independent) for a folding pattern of eight β strands. At each iteration, nevertheless, the algorithm given by equations (6)–(8) looks through this multitude of structures and their interactions with a field. This takes no more than $8 \times (10^2 \times 10 \times 5)^2 \sim 10^8$ operations. The iterative search of a self-consistent field converges rather fast (in 4–8 steps, 10–20 min on an IBM 370). The solutions prove to be independent of the starting 'field'. This implies that a field is rather simple: indeed, it looks like a hydrophobic drop pierced by the lattice (Figs 1 and 2), and only the shape of this drop is refined.

First, it was checked whether we can find the positions of strands in space and sequence for γ -crystallin, provided that the native folding pattern is given. The calculated structure is close to the native one (Fig. 3). Substantial deviations were

FIG. 3 The calculated location of β strands in space and in the sequence of γ -crystallin. The numbers denote the average positions of strand termini and ranges of fluctuations. Because of fluctuations, the average positions of termini are between the lattice points. The fluctuations are partly due to the movement of a strand as a whole but mainly to the end residues which are added or deleted whereas the positions of internal ones stay the same. List of parameters F_β and ΔF_β (kcal mol $^{-1}$) used for calculation: Val -0.8 , $+0.5$; Ile -0.8 , $+0.5$; Phe -0.7 , $+0.5$; Tyr -0.6 , $+0.4$; Trp -0.45 , $+0.5$; Leu -0.4 , $+0.5$; Met -0.4 , $+0.4$; Cys -0.4 , $+0.4$; Thr -0.3 , $+0.3$; His -0.2 , $+0.3$; Gln 0 , $+0.2$; Ala 0 , $+0.3$; Ser $+0.05$, $+0.25$; Lys $^+$ $+0.2$, $+0.1$; Arg $^+$ $+0.2$, $+0.05$; Glu $^-$ $+0.2$, $+0.05$; Asn $+0.3$, $+0.15$; Asp $^-$ $+0.4$, -0.05 ; Gly $+0.7$, -0.1 ; Pro $+1.0$, -0.2 (ring in) or -1.4 (ring out). Some of these parameters are estimated from thermodynamics (F_β) and kinetics (ΔF_β) of β folding in polypeptides, and others have been calculated^{23,25,26}. The loops must be ≥ 1 residue long for one such as loop 2–3 and ≥ 3 for one such as loop 3–4. A term $\Delta u = RT \ln [1 + 0.05 \exp(-\Delta G_{np}/RT)]$ is added to u_{ii+1} for each residue of an internal loop (3–4, 4–5 and 7–8). This accounts for a greater immersion of these loops²⁵. Other loop interactions are ignored.

found mostly for the edge β strands of the sheets. Thus, assuming the precise topology, we can calculate the precise distribution of the sequence among this fold, and its precise structural arrangement.

Second, we tried finding the native fold, given the amino-acid sequence of some β -sandwich domains. All four examples have eight antiparallel strands, but they represent three different folding patterns. The most stable fold is searched for only among the 60 'potentially stable' patterns mentioned above (we assume that the native fold must be a β sandwich), using the energy terms described in Figs 2 and 3. For each chain and each of these 60 topologies, we calculate again the secondary structure, its arrangement, and the corresponding partition function. The result (Fig. 4) is that for each sequence the native folding pattern is among the three most stable ones calculated, and despite the coarse parameters of the model nearly all other patterns are strictly rejected. Analysis of the results (see also ref. 21) implies that a three-dimensional fold is stable only if it is 'consistent' with the amino-acid sequence: (1) the order of inner and edge β strands (those strands deeply versus those slightly immersed in the molecular field) matches the order of the more and the less hydrophobic regions in the chain; (2) strands which are close in space have hydrophobic surfaces similar in size; (3) the loops required by a folding pattern correspond to the lengths of hydrophilic regions. $\square$

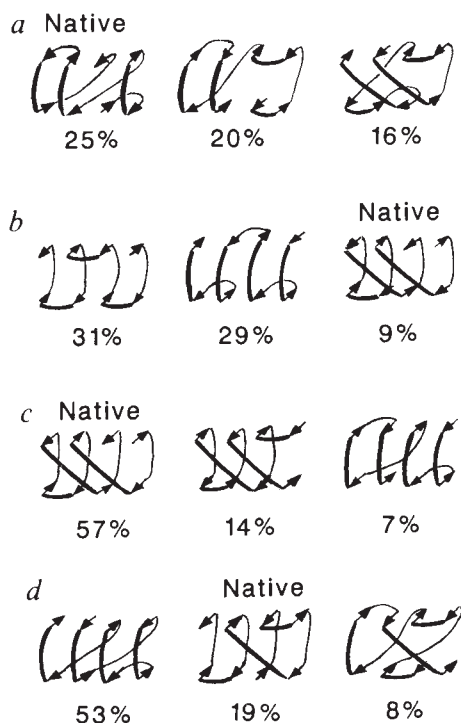


FIG. 4 Calculated thermodynamic probabilities, $[(Z_{\text{fold}}/(\sum_1^{60} Z_{\text{fold}}))]100\%$, for amino-acid sequences of different β domains. Only three most stable folding patterns from a set of 60 possible are shown; an average probability for each of the other 57 is $\sim 0.5\%$. a, γ -crystallin, N domain²²; b, catabolite activator protein, β domain²⁷; c, satellite tobacco necrosis virus²⁸; d, human pancreatic lipase, β domain²⁹.

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Humanized antibodies for therapy

Man Sung Co and Cary Queen

Protein engineering has made it possible to combine the binding sites of murine antibodies with human antibody regions. Antibodies constructed in this way have important advantages for therapy.

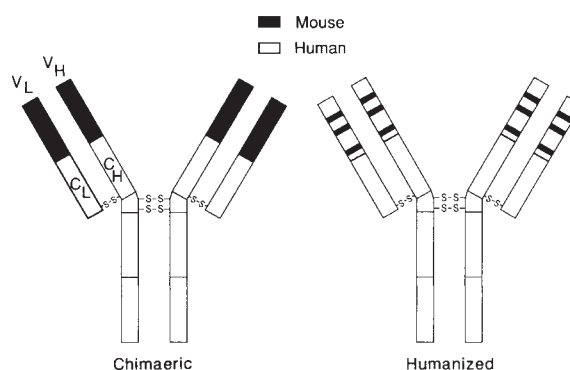
ANTIBODY therapy holds great promise for the treatment of cancer, autoimmune disorders, and viral or bacterial infections. Antibodies can neutralize microorganisms and their toxins, destroy cancer cells, and modulate the immune system. Murine monoclonal antibodies are relatively easy to produce but are severely restricted for therapeutic use by their immunogenicity in patients. Human monoclonal antibodies may function better therapeutically but have been very difficult to generate. A possible solution is the use of genetic engineering to construct humanized antibodies, which combine the binding sites from murine antibodies with human antibody elements. Ideally, a humanized antibody will retain the high binding affinity of the mouse antibody while achieving the therapeutic advantages of a human antibody: that of improved effector function, longer half-life and, most importantly, reduced immunogenicity.

Advantages of humanized Abs

When a murine antibody is used to treat patients, there is generally a strong immune response against the injected antibody¹, which precludes long-term treatment. This human anti-mouse antibody (HAMA) response may potentially be reduced by transforming the murine monoclonal antibody into a chimaeric antibody (Fig. 1). Such an antibody combines the entire variable (V), or binding, domain of a mouse antibody with a human antibody constant (C), or Fc, domain². However, when the murine antibody OKT3 is used in patients, much of the antibody response is directed against the V region rather than the C region³. Hence chimaeric antibodies, in which the V region remains murine, may still be immunogenic. In fact, the immunogenicity of different chimaeric antibodies seems to vary, with one chimaeric antibody substantially non-immunogenic and a second almost as immunogenic as the original mouse antibody⁴. One may speculate that a chimaeric antibody will be more immunogenic if its light or heavy chain V region is not very homologous to any human V region.

Conceptually, it is possible to go a step further in reducing the immunogenicity of a murine antibody, by utilizing the fact that any antibody V domain consists of six complementarity-determining regions (CDRs) embedded in a framework region. The CDRs fold up to contact directly the antigen, while the framework holds the CDRs in position. Winter and colleagues⁵, therefore, chose to introduce only the minimal binding

FIG. 1 A chimaeric antibody retains the entire V domain from the murine antibody, whereas a humanized antibody retains only the complementarity-determining regions.



site, the CDRs, from a rodent antibody into a human V-region framework, combined with a human C region (see Fig. 1). Antibodies made in this way have been called reshaped, CDR-grafted or humanized. (Unfortunately, some authors call any chimaeric antibody 'humanized'.)

The first fully humanized antibody⁵, CAMPATH-1H, which binds to an antigen on lymphocytes, has been used to treat two patients with non-Hodgkin lymphoma⁶ and one patient with systemic vasculitis⁷. No antibody response against CAMPATH-1H was detected in these three patients and the treatment induced disease remissions. The anti-Tac antibody, which binds to the human IL-2 receptor, has also been humanized⁸. Humanized anti-Tac has potential as an immunosuppressive drug because it inhibits proliferation of T cells by blocking IL-2 binding. Treatment with humanized anti-Tac significantly prolonged the survival of cardiac allografts in cynomolgus monkeys⁹. None of the monkeys receiving humanized anti-Tac produced measurable antibodies against it before day 33, whereas animals receiving murine anti-Tac developed a strong antibody response on mean day 11. These early results suggest that humanized antibodies will be substantially less immunogenic in humans than murine and perhaps chimaeric antibodies.

Two other potential advantages of humanized antibodies are improved effector function and longer circulating half-life in patients. The CAMPATH-1H antibody is more effective at mediating antibody-dependent cellular cytotoxicity with human effector cells than the mouse antibody⁵. Humanized anti-Tac is able to mediate antibody-dependent cellular cytotoxicity against human T cells, despite the absence of such activity by murine anti-Tac¹⁰. And, humanized anti-Tac has a much longer half-life in monkeys than murine anti-Tac⁹.

Because effector function and half-life largely depend on the Fc region of an antibody, these properties are also often improved in chimaeric antibodies.

Design of humanized Abs

The key step in constructing a humanized antibody is to choose sequences for its light and heavy chain V regions — the rest is genetic engineering. Despite initial success¹¹, in our experience and that of others, it is rarely sufficient to combine the CDRs from the murine antibody with a completely human framework. That is because certain residues in the original murine framework make key contacts with the CDRs that help maintain their conformation. When the murine framework is replaced with a human sequence, those contacts are altered, distorting the shape of the CDRs and reducing or abolishing their affinity for antigen.

Two approaches have been used to solve this problem. In our approach, human V regions that are especially homologous in sequence to the murine V regions are chosen from a data base in order to provide the human framework. Our thinking is that there is no *a priori* reason to prefer one human antibody over another, so one might as well use V regions that are more similar to the mouse V regions and thus less likely to distort the CDRs. Second, computer modelling of the antibody is used to identify the few mouse residues that make key contacts with the CDRs, and those amino acids are introduced into the human framework along with the CDRs themselves. Using this method, we have successfully humanized the anti-Tac antibody, antibodies against the gB and gD glycoproteins of herpesvirus (HSV)¹², and several other antibodies. In each case, the first construct made retained high binding affinity, and the humanized anti-HSV antibodies retained full viral neutralizing activity¹².

Other researchers have chosen a different approach, as illustrated by the humanization of CAMPATH-1H and an anti-respiratory syncytial virus antibody¹³. The same human V regions are always used to provide the framework, and initially only the murine CDRs are grafted onto it. When it is found that the humanized antibody has reduced or no binding affinity, new constructs are made that incorporate one to three additional mouse residues near the CDRs, and so on iteratively until binding is restored. This method minimizes the number of murine residues at the expense of some trial and error, and may not recover the full biological activity of the original antibody¹³.

To date, one humanized antibody has been used to treat patients. Humanized anti-Tac will begin clinical trials soon, and humanized anti-HSV and anti-RSV antibodies are being tested in animals. The hope is that humanized antibodies may ultimately fulfill the promise of antibody therapy. □

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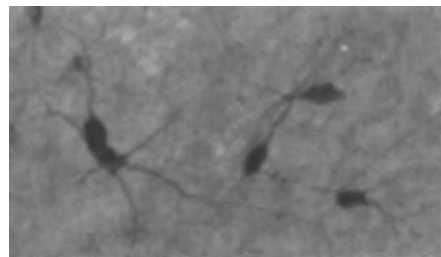
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Reader Service No. 50

Immunology briefs

Neuropeptide Y antiserum for immunohistochemical use, a monoclonal antibody to cytokeratin 20 and a range of fluorescent microspheres for antibody attachment — new products for today's immunologist.

INCSTAR, offers a **rabbit polyclonal antiserum to neuropeptide Y**, which is intended for use in research studies of the central and peripheral nervous system, and studies of endocrine tumours such as pheochromocytomas and neuroblastomas (*Reader Service*



Cell bodies in rat cortex labelled with INCSTAR's neuropeptide Y antiserum using peroxidase anti-peroxidase staining.

No. 101). The antiserum has been used to detect neuropeptide Y in a wide range of species — fish, newt, frog, bird, rat, cat and human. Anti-neuropeptide Y produces excellent staining results with both fluorescent and enzyme labelling methods, says INCSTAR. The antiserum shows no cross-reactivity with members of its family that have marked sequence homology, namely peptide YY and pancreatic polypeptide.

Cymbus Bioscience has added the monoclonal antibody to Cytokeratin 20 to its range of **cytokeratin antibodies** for immunohistology (*Reader Service No. 102*). The monoclonal antibody to Cytokeratin 20 can be used for the detection of gastrointestinal adenocarcinomas, transitional cell carcinomas and Merkel cell tumours. The antigen recognised is a polypeptide with a molecular weight of 46,000. The antibody is suitable for use with frozen sections, formalin-fixed paraffin wax-embedded tissues, cytological material and western immunoblots.

LPS-Rinse, a new **reagent for the selective removal of endotoxins from antibody preparations**, is now available from a company of the same name (*Reader Service No. 103*). Potential endotoxin sources include water, media, chemicals, glassware and the chromatography media used in antibody production. When present, endotoxins can be stimulatory for stem cells or cytotoxic for other cell types, says the company. In addition, endotoxin removal can be complicated by the charge, size heterogeneity and tendency of endotoxins to adhere to proteins. LPS-Rinse is a non-detergent formulation that is added at low concentrations to chromatography buffers used in antibody purification.

According to Nycomed, the three-step procedure of the NycoCard C-Reactive Protein (CRP) **immunoassay** can be used to determine CRP concentration levels in blood or seum in two minutes (*Reader Service No. 104*). In the immunoassay, 25 µl of whole blood or 20 µl of serum is applied to one of six holes on the test card. The CRP molecules are trapped on the underlying membrane carrying CRP-specific monoclonal antibodies. A drop of gold particles is added and membrane-bound CRP is identified by the reddish-purple colour of the gold particles. Excess conjugate can be removed by a drop of washing solution. CRP concentration levels can be determined by comparing the intensity of the coloured test response with a reference chart that has five CRP concentration levels ranging from 10 to 200 milligrams per litre. Two further tests, NycoCard U-Albumin and NycoCard D-Dimer, will be available in autumn.

Non-isotopic detection

Kirkegaard & Perry Laboratories has introduced a new TMB Microwell Peroxidase Substrate that offers the convenience of a **single-solution substrate** and produces an intense blue colour in the presence of peroxidase-labelled antibodies (*Reader Service No. 105*). The rapid reaction rate permits



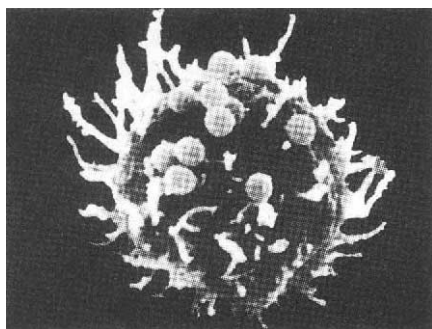
K & P's single solution peroxidase substrate for microwell plate assays.

accurate, quantitative measurement in kinetic ELISA studies (max = 650 nm), says K & P. The sensitivity can be enhanced further for endpoint ELISA determinations (max = 450 nm) by the addition of phosphoric acid, which converts the blue chromophore to yellow. The TMB Microwell Peroxidase Substrate kit provides 400 ml of working solution. K & P also offers TMB Membrane Peroxidase Substrate, which produces bands of blue colour at membrane sites bearing peroxidase. The reagent, which is intended for the detection of immobilized proteins produced by western transfer or dot blots, is supplied in a kit that produces 200 ml of working solution.

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Bio-Rad has come up with the Immun-Lite assay kit for the **detection of specific antigens on membranes** in picogram amounts (*Reader Service No. 106*). The kit is based on non-isotopic chemiluminescent technology and uses a new chemiluminescent substrate, adamantyl-1,2, dioxetane phosphate (AMPPD), which emits light when enzymatically activated by alkaline phosphatase. The light energy can then be captured on either X-ray film (with the option of quantification by densitometry) or instant photographic film. Chemiluminescent detection can produce a strong positive signal on film after only 1–15 minutes, says Bio-Rad, as compared to the overnight exposures required with ^{125}I . In addition, as the light emission is sustained over many hours, researchers can try out different exposure times.

Three types of Covasphere **fluorescent microspheres**, immunoreagents that are designed for the attachment of antibodies or other proteins, have been developed by Cella (*Reader Service No. 107*). MX Covaspheres feature a surface that forms covalent bonds with antibodies at room temperature and neutral pH, without the need to use chemical coupling agents, says Cella. They are intended for use primarily as markers for cell surface antigens in flow cytometry and fluorescence microscopy. CX Covaspheres are designed for use in studies of phagocytic cells and can be observed in cell tissue using epifluorescence microscopy. FX Covaspheres can be covalently bound to carboxylate groups on proteins and other ligands using a coupling agent. Green, red, blue and non-fluorescing Covaspheres are available in suspension.



Human myeloid cell labelled with Cella's CX Covaspheres using My-1 mAb.

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Cytokine selection

Cambio has introduced a new **polyclonal antibody to IL-5**, a high-titre (2,000:1) rabbit antiserum that is specific for a single IL-5 epitope (*Reader Service No. 108*). The polyclonal antibody is recommended for use in ELISAs and has been used to detect quantities of IL-5 as low as 50 ng, says Cambio. It does not cross-react with murine IL-5 and is not immunoprecipitating. A second polyclonal antiserum, raised to a different epitope of the interleukin, and an IL-5 monoclonal will be released shortly.

Purified **human recombinant IL-4**, isolated from *Escherichia coli* strains bearing plasmids with interleukin structural genes, has been developed by Janssen Biochimica (*Reader Service No. 109*). Human recombinant IL-4 is biologically active and has a specific activity of 10^6 units per milligram, says Janssen (where one unit is the quantity of human recombinant IL-4 at which there is 50 per cent of maximum incorporation of ^3H -thymidine). The homogeneity of the purified human recombinant IL-4 is tested by SDS-PAGE and HPLC.

Endogen is offering a host of **monoclonal antibodies to murine cytokines** (*Reader Service No. 110*). The line-up includes: anti-murine IL-2, anti-murine IL-3, anti-murine IL-4, anti-murine IL-5, anti-murine IL-6, anti-murine TNF alpha, anti-murine interferon gamma and anti-murine GM-CSF. Anti-murine IL-3, IL-4, IL-5, IL-6, TNF and GM-CSF are available in pairs as IgG1 and/or IgG2 monoclonals for use in, for example, western blot analysis and neutralization studies. Anti-murine IL-2 is an IgG2 monoclonal antibody and anti-murine interferon gamma is an IgG1 monoclonal. Both antibodies can be used in blocking studies and in western blot analysis. All of the above are available without preservatives in 500- μg vials at 1 mg ml^{-1} in a sterile, carrier-free liquid.

R & D Systems has a new line of **murine cytokine reagents** that includes the purified recombinant cytokines IL-1 beta, IL-3, IL-4, IL-5, IL-6, IL-7, TNF alpha, GM-CSF and MIP-1 alpha, and neutralizing polyclonal antibodies directed against murine IL-1 beta, IL-3, IL-4, IL-6, IL-7 and GM-CSF (*Reader Service No. 111*). All of the cytokines are tested for biological activity and supplied as lyophilized reagents with greater than 95 per cent purity, says R & D Systems; carrier-free cytokines, supplied frozen in solution, can be purchased at no extra cost. The neutralizing antibodies, which are raised in goats, are supplied lyophilized as one milligram of Protein G-purified IgG.

Research tools

Schleicher and Schuell has introduced a complete line of Affinica **affinity chromatography media** on a new polymeric support that has been designed to withstand the high flow rates of automated chromatography systems (*Reader Service No. 112*). Coupled to this polymethylmethacrylate support are Affinica Ig binding ligands. Affinica Protein A Polymeric Support uses a recombinant truncated Protein A molecule that allows more Fc binding domains to be immobilized per millilitre of gel than other Protein A supports, says S & S. In addition, the company offers Affinica Protein G, which is specific for IgG, and the versatile Affinica Protein A/G, a chimaeric molecule that has the binding domains of both Protein A and Protein G. Completing the line-up is the

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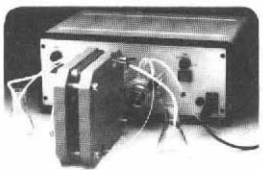
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The Miniblotter 12 from Integrated Separation Systems is designed for the screening of antibodies and/or antigens using minimal amounts of reagents (*Reader Service No. 113*). As the Miniblotter has been engineered to match the lanes in intermediate Daiichi gels and Mini-Septrags, it is now possible to run antigen in the twelve lanes of the intermediate gel and then exactly match those lanes to the twelve channels of the miniblotter. There is no need to cut membranes and all of the Miniblotter's channels can be washed simultaneously, says ISS.

Researchers in the Department of Immunology at the University of Aarhus have designed two types of disposable tray for use in western blot staining, ELISAs, cell culture, hybridoma technology and anti-virus antibody screening (*Reader Service No. 114*). The trays are made of a polypropylene material in order to minimize the adsorption of proteins and to withstand autoclaving. Two tray designs are available. Octaline trays, which have eight troughs, can be used for staining membrane strips in western blot and dot blot procedures. The trays are designed to allow the minimum use of reagent per strip (typically 0.5 or 1.0 ml). In addition, the Octaline trays can be used as reagent reservoirs in ELISAs where different reagents or reagent concentrations are used for each row. Polystyrene lids to the trays are optional extras. Octatray disposable V-shaped reagent reservoirs for use with 8- or 12-channel multipipettes have a 100-ml capacity. They are intended for use in ELISAs and cell culture research.

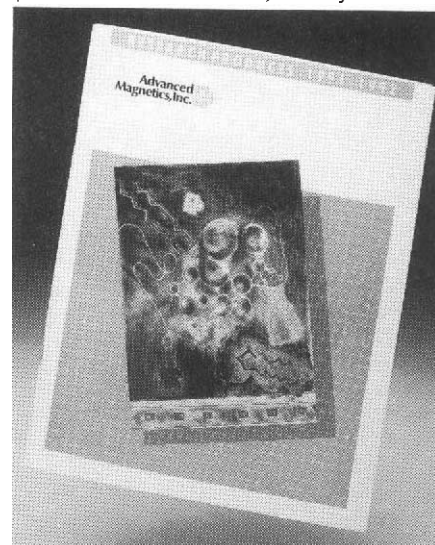
Information sources

LIFO-DISK (Liposome Information Electronic Liposome Library) is a data base of papers published on liposome research from MM Developments (*Reader Service No. 115*). With LIFO-DISK, users can access all the recorded titles dating back to 1965 that contain specific keywords, or those published in a particular year or by a particular author. In addition, users may file selected titles for use as references in publi-

cations or mark selected titles for printing or word processing. The data base, which requires an IBM PC or compatible, is updated every three months and costs \$390 (US) or \$460 (Canada). An Apple Macintosh version will be available shortly.

The new 48-page 1991 catalogue from Biomedica, which contains sections on immunocytochemistry, flow cytometry, immunochemicals and molecular biology, is now available upon request (*Reader Service No. 116*). The immunocytochemistry section contains a review of avidin-biotin complex technology and details of the company's new Autoprobe III and Ultraprobe detection systems. Histoscan and BioStain kits are available in peroxidase and alkaline phosphatase, in addition to phycoerythrin for fluorescent techniques. Fluorescent avidin-biotin reagents, affinity purified antibodies, and lectins, make up the section on flow cytometry. A selection of immunochemicals includes affinity purified antibodies to a variety of immunoglobulins of different animal species, and conjugates of these antibodies to biotin, peroxidase and alkaline phosphatase. This section also includes lectins and ready-to-conjugate SPDP- (*N*-succinimidyl 3-(2-pyridyldithio) propionate) labelled proteins.

The 1991-1992 catalogue of research products from Advanced Magnetics is hot off the press and available free upon request (*Reader Service No. 117*). Thirty five RIA



New product news from Advanced Magnetics.

and EIA kits, over 70 BioMag products, and a wealth of immunoassay reagents are featured in the catalogue. In addition, protocols are included for the collection, preparation and extraction of eicosanoid and atrial natriuretic peptide samples. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.